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Short Communication

Automated on-line column-switching high performance liquid chromatography isotope dilution tandem mass spectrometry method for the quantification of bisphenol A, bisphenol F, bisphenol S, and 11 other phenols in urine

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ABSTRACT

Human exposure to bisphenol A (BPA) is widespread. However, in recent years, bisphenol analogs such as bisphenol S (BPS) and bisphenol F (BPF) are replacing BPA in the production of some consumer products. Because human exposure to these alternative bisphenols may occur, biomonitoring of these bisphenol analogs is warranted. In the present study, we developed and validated a sensitive and selective method that uses on-line solid phase extraction coupled to high performance liquid chromatography-isotope dilution tandem mass spectrometry with peak focusing to measure BPA, BPF, BPS, and 11 other environmental phenols in urine. The method required a small amount of sample (100 μL) and minimal sample pretreatment. The limits of detection were 0.03 ng/mL (BPS), 0.06 ng/mL (BPF), 0.10 ng/mL (BPA), and ranged from 0.1 ng/mL to 1.0 ng/mL for the other 11 phenols. In 100 urine samples collected in 2009–2012 from a convenience group of anonymous adults in the United States, of the three bisphenols, we detected BPA at the highest frequency and median concentrations (95%, 0.72 ng/mL), followed by BPS (78%, 0.13 ng/mL) and BPF (55%, 0.08 ng/mL). This sensitive, rugged, and labor and cost-effective method could be used for the analysis of large number of samples for epidemiologic studies.

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1. Introduction

Human exposure to bisphenol A (BPA) is widespread due to the extensive use of this chemical in consumer products [1]. Scientific debate continues about the toxicity of BPA in animal studies and its potential implications for human health [2], and government organizations in several countries have banned the use of BPA in certain products [3–5]. In response to these restrictions and public pressure, the use of bisphenol analogs, including bisphenol S (BPS, 4,4′-sulfonyldiphenol) and bisphenol F (BPF, 4,4′-dihydroxydiphenylmethane), may be increasing.

Limited data suggest that BPS and BPF, similar to BPA, possess slight to moderate acute toxicity and weak estrogenicity, although the activity of BPF and BPS seems to be lower than that of BPA [6,7]. Nonetheless, the potential environmental and health impacts of BPF and BPS are largely unknown, and human exposure data to these compounds are warranted.

Several analytical techniques involving extraction, purification and/or derivatization followed by detection with gas chromatography–mass spectrometry, high-performance liquid chromatography–tandem mass spectrometry (HPLC–MS/MS) or ultra performance liquid chromatography have been used to measure BPF and BPS in sediments, paper products, and food [8–12]. Analytical methods to measure BPF and BPS in biological fluids, on the other hand, are rather limited. BPS was recently quantified by HPLC–MS/MS in urine after automated off-line solid-phase extraction (SPE) [13].

In the present study, we developed and validated a sensitive and selective method that uses on-line SPE–HPLC–MS/MS with peak focusing to measure the urinary concentrations of BPA, BPF, and BPS. The same method can be also used to simultaneously measure 11 other phenols: 2,4- and 2,5-dichlorophenols; 2,4,5- and 2,4,6-trichlorophenols; o-phenylphenol, benzophenone-3, triclosan, and methyl-, ethyl-, propyl- and butyl parabens. These environmental phenols are used in consumer and personal care products and human exposure to these chemicals is of public health interest.

Abbreviations: CDC, Centers for Disease Control and Prevention; BPA, bisphenol A; BPF, bisphenol F; BPS, bisphenol S; HPLC, high performance liquid chromatography; LOD, limit of detection; On line-SPE, on line-solid phase extraction; QC, quality control; QCH, quality control high; QCL, quality control low; QCM, quality control medium; RAM, restricted access materials; RSD, relative standard deviation.

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2. Experimental

2.1. Analytical standards and reagents

BPA, BPF, and BPS were purchased from Sigma-Aldrich Laboratories, Inc. (St. Louis, MO, USA). $^{13}C_{12}$-BPA was obtained from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA) and $^{13}C_{12}$-BPS from CanSyn Chem Corp. (Toronto, Canada). The sources of the other standards and reagents are given in section 1 of the Supplemental Material.

2.2. Collection of human urine for method validation

We collected 100 urine samples in Atlanta, GA in 2009–2012 from 100 adult volunteers with no documented occupational exposure to the target phenols. The Centers for Disease Control and Prevention's (CDC) Human Subjects Institutional Review Board reviewed and approved the study protocol. A waiver of informed consent was requested under 45 CFR 46.116(d). No personal or demographic data were available.

2.3. Preparation of standard stock solutions and quality control materials

We prepared the stock solutions of individual analytical standard and stable isotope-labeled internal standard in MeOH. Ten mixed stock solutions containing all 14 analytes were generated by serial dilution of the individual stock with MeOH. The final concentrations of the mixed stock standards ranged from 0.01 to 100 ng/mL for BPF and BPS, and from 0.1 to 100 (or 1000) ng/mL for BPA and the other 11 analytes. The mixed internal standard solution, containing the stable isotope-labeled analogs for all target analytes except BPF, was prepared so that a 50 μL spike would result in a concentration of 25 ng/mL; we used $^{13}C_{12}$-BPA as the internal standard for BPF. We prepared a second set of individual stock and mixed stock standards and used them to fortify quality control (QC) materials and to check the method accuracy. The mixed stock solutions and mixed internal standard solution, dispensed into 1.5 mL glass vials and 10 mL glass vials respectively, were stored at −70 °C until used.

QC materials were prepared from blank urine pre-screened to confirm that it did not contain detectable concentrations of the target analytes. The blank urine was divided into two aliquots to create QC low (QCL) and QC high (QCH) concentration pools. The QCL and the QCH pools were enriched with different levels of native target compounds that encompass the ranges described for the U.S. general population [13,14]. All QC materials were stored in 1.5 mL glass vials at −70 °C until used.

The enzyme solution was prepared daily as described in section 2 of the Supplemental Material. A mixture of $^{13}C_4$-4-methylumbelliferone, 4-methylumbelliferyl sulfate, and 4-methylumbelliferyl glucuronide was added to each sample and used as a deconjugation standard to confirm that the enzyme functioned properly. Additional details are given in the Supplemental Material.

2.4. Sample and standard preparation

To measure both the concentration of free and total (free plus conjugated) species of the target phenols, each unknown sample was prepared in two different ways: one sample was processed without enzyme treatment; the other was treated with β-glucuronidase/sulfatase. For estimating the concentrations of free species, we added 50 μL of internal standard solution and 100 μL of urine in a 1.5 mL conical silanized glass autosampler vial, and diluted the urine to 1 mL with 0.1 M formic acid. For measuring the total concentrations, we added 50 μL of internal standard solution, 50 μL of deconjugation standard, and 50 μL of β-glucuronidase/sulfatase to 100 μL of urine in a conical silanized glass autosampler vial. After being gently mixed, the urine was incubated at 37 °C for 4 h. Upon incubation, 750 μL of 0.1 M formic acid was added. All samples were vortex mixed and centrifuged at 812 g for 15 min before the on-line SPE–HPLC–MS/MS analysis.

We prepared standards, QCs, and reagent blanks daily for each batch using the same procedure as described above for study samples to be analyzed for total concentrations, but replaced the urine with the same volume of mixed standard stock solution, QC materials, and HPLC-grade H_2O (reagent blank). In addition to study samples, each batch includes analytical standards, QCH, QCL and reagent blanks. Calibration curves must have $r^2 > 0.99$. The concentrations of the QC materials are evaluated using standard statistical probability rules [15]. All laboratory operations are conducted under the requirements set forth in the Clinical Laboratory Improvement Act of 1988.

2.5. On-line SPE–HPLC–MS/MS with peak focusing

The on-line SPE–HPLC–MS/MS system was modified from previous methods [16,17], and consisted of several Agilent 1200 modules (Agilent Technologies, Wilmington, DE, USA) and an ABSciex 5500 QTRAP mass spectrometer (Applied Biosystems, Foster City, CA, USA) equipped with an atmospheric pressure chemical ionization (APCI) interface. The SPE column was LiChrospher™ RP-18 ADS (25 × 4 mm, 25 μm particle size, 60 Å pore size; Merck KGaA, Germany) and the HPLC column was Chromolith® High Resolution RP-18e (100 × 4.6 mm, Merck KGaA, Germany). The injection volume was 350 μL.

We designed the on-line SPE–HPLC–MS/MS system to allow for concurrent SPE and HPLC–MS/MS cycles with peak focusing (i.e., diluting the SPE elute before HPLC) as previously described [16]. The on-line SPE procedure details, the negative fragment ions used for quantification and confirmation and the retention times for all analytes are given in the supplemental material (Section 3, Tables S1–S2).

3. Results and discussion

3.1. On-line extraction of urine with restricted access material (RAM)

We used RAM, a type of extraction sorbent used for on-line sample clean up and extraction of biological samples [18]. As a result, the urine pre-treatment was minimal and simply involved the addition of internal standard, deconjugation with β-glucuronidase/sulfatase, and dilution with formic acid, followed by centrifugation. The SPE recoveries of the 14 phenols from urine were calculated as the ratio of response factors calculated from two experiments by adding internal standard before or after SPE (Section 4, supplemental material). The calculated SPE recoveries ranged from 77% to 106% (supplemental material, Table S3) suggesting adequate extraction of the target compounds from the urine matrix.

3.2. Method validation and quality control

Pre-screened blank urine spiked with standard and isotope-labeled internal standard solutions was analyzed repeatedly to determine the limits of detection (LODs). LODs were calculated as $3S_0$, where S_0 is the standard deviation as the concentration approaches zero [19]. S_0 was determined from measurements of three low-level standards prepared in urine. The calculated LODs of the three bisphenols were 0.1 ng/mL (BPA), 0.06 ng/mL

Table 1
Accuracy, limits of detection (LOD), and inter- and intra-day precision.[a]

Analyte	Accuracy			LOD[b] (ng/mL)	Precision			
	Recovery (%) (spiked concentration in ng/mL)				QC high		QC low	
	(1)	(5)	(10)		Mean Conc. (ng/mL)	RSD (%)	Mean Conc. (ng/mL)	RSD (%)
BPA	104	102	99	0.1	9.9	5.9	2.1	5.4
Benzophenone-3	86	106	105	0.2	50.9	5.5	12.6	7.5
Triclosan	108	103	99	1.0	67.8	8.1	23.5	9.9
2,4-Dichlorophenol	118	99	102	0.1	8.8	5.3	1.8	7.5
2,5-Dichlorophenol	83	101	109	0.1	53.0	5.0	5.8	6.1
2,4,5-Trichlorophenol	102	103	99	0.1	12.7	4.6	2.5	5.7
2,4,6-Trichlorophenol	115	94	101	0.1	19.7	5.9	6.6	9.7
o-Phenylphenol	101	104	99	0.1	9.5	5.1	1.9	6.5
Methyl paraben	99	104	105	0.1	49.6	4.4	5.7	5.9
Ethyl paraben	106	107	106	0.1	20.5	5.0	4.6	7.9
Propyl paraben	107	106	105	0.1	19.9	6.8	4.5	5.6
Butyl paraben	103	106	102	0.1	9.5	5.5	2.1	4.7
BPS	107	104	105	0.03	4.9	6.4	0.5	6.1
BPF	91	102	103	0.06	5.0	6.7	0.5	12.1

[a] Relative standard deviation (RSD %) was calculated from 50 repeated measurements of the quality control (QC) materials.
[b] We used 0.1 ng/mL as the working LOD for all the other 11 phenols except triclosan and benzophenone-3, because the concentration of the lowest standard was 0.1 ng/mL for these phenols.

(BPF), and 0.03 ng/mL (BPS); for the other 11 phenols, LODs were 0.1 ng/mL, except for triclosan (1.0 ng/mL) and benzophenone-3 (0.2 ng/mL) (Table 1). The LOD of BPS is comparable to that reported recently (0.02 ng/mL), but our method required less urine (100 μL vs. 500 μL) [13]. Furthermore, the current method provided better sensitivity than our previous methods, with LODs of BPA and most of the other phenols 2–5 times lower than those reported previously, even with a smaller injection volume (350 μL vs. 900 μL) [16,17]. Carryover was <0.05% for all analytes.

The method accuracy was assessed by analyses of blank urine spiked at 10 concentrations covering the entire calibration range (Supplemental Material, Table S4). Accuracy, expressed as the percentage of the recovery, from three selected spiking levels (1, 5, and 10 ng/mL), ranged from 86 to 118% for all analytes (Table 1). The stability of analytes in the mixed stock solutions was evaluated by comparing the concentrations of 100 urine samples, calculated from two standard calibration curves constructed with recently prepared or three year old (stored at −70 °C and having undergone several freeze-thaw cycles) mixed stock solutions. The concentrations obtained using the two sets of standards were indistinguishable suggesting no degradation of the target analytes in the old stock standards.

We determined the method precision from 50 repeated measurements of the two QC materials over 6 months (Table 1). The relative standard deviations (RSDs), which reflect the intra- and inter-day variability of the method, ranged from 4.4% to 12.1% for all of the analytes (Table 1). These precision data suggest that, under our experimental storage and analysis conditions, the target analytes were stable in urine for at least six months. Furthermore, our previous research [20] showed that BPA and the other 11 phenols in urine could be stable for at least up to 30 months when stored at −70 °C. We have no reason to believe that BPS and BPF would be any different under the same experimental conditions [20].

We evaluated the matrix effects by calculating the matrix factor, defined as the ratio of analyte peak area in the presence of urine to the analyte peak area in the absence of urine. The matrix factors, calculated from three spiking concentrations (0.5, 5, 10 ng/mL), ranged from 89 to 138% depending on the analyte (supplemental material Table S3). We also compared calibration curves prepared from analytical standards spiked in H_2O and six different urine matrices. The slopes of the calibration curves in H_2O or urine were quite similar (i.e., percentage of the difference <5%, calculated from the mean slope of three sets of standards prepared in H_2O and in each urine), suggesting that the accuracy of the method was not compromised by the matrix. We used H_2O-based curves for quantification. The calibration curves, constructed using $1/x$ weighing linear regression, showed adequate linearity with correlation coefficients greater than 0.99 for all analytes.

The Chromolith® High Resolution RP-18e, a second generation of monolithic type column, provided higher HPLC resolution than a regular monolithic column. Compared with our previous methods [16,17], we achieved better peak shapes and shorter run time even measuring two additional analytes, BPF and BPS, using one high resolution column instead of two regular monolithic columns in tandem. Typical HPLC–MS/MS chromatograms are shown in Fig. 1.

3.3. *Quantification of BPA, BPF, BPS and 11 other phenols in human urine*

We tested the usefulness of the current method by analyzing 100 urine samples collected between 2009 and 2012 from 100 adult volunteers. Because the target phenols can be used in a variety of consumer and personal care products, and we used these archived urine samples for previous projects, we also determined the concentration of conjugated phenols by subtracting the respective concentrations of free species from the total concentrations. The mean, median, range of concentrations, detection frequency and mean percentage of conjugated species (calculated as the ratio of concentrations of conjugated and total species) of the three bisphenols are presented in Table 2; the total urinary concentrations of the other 11 phenols (supplemental material, Table S5) were within published ranges [14,16,17]. The concentrations of BPA and the other 11phenols in these 100 samples obtained using our previous methods [16,17] and the current method showed strong correlations (e.g., $R^2 > 0.98$ for the linear correlation plot with slopes and intercepts that were not statistically different ($p > 0.05$) from 1 and 0, respectively).

Among the bisphenols, we detected BPA at the highest frequency and median concentrations (95%, 0.72 ng/mL), followed by BPS (78%, 0.13 ng/mL) and BPF (55%, 0.08 ng/mL) (Table 2). The detection rate of BPA was similar to that reported for the US general population [14]. The mean conjugate percentage of BPA, BPF and BPS ranged from 97 to 100% (Table 2) suggesting that the conjugated forms are the major urinary species of the bisphenols, in agreement with previous reports, mainly on BPA [21]. These data also suggested that minimum, if any, external contamination with

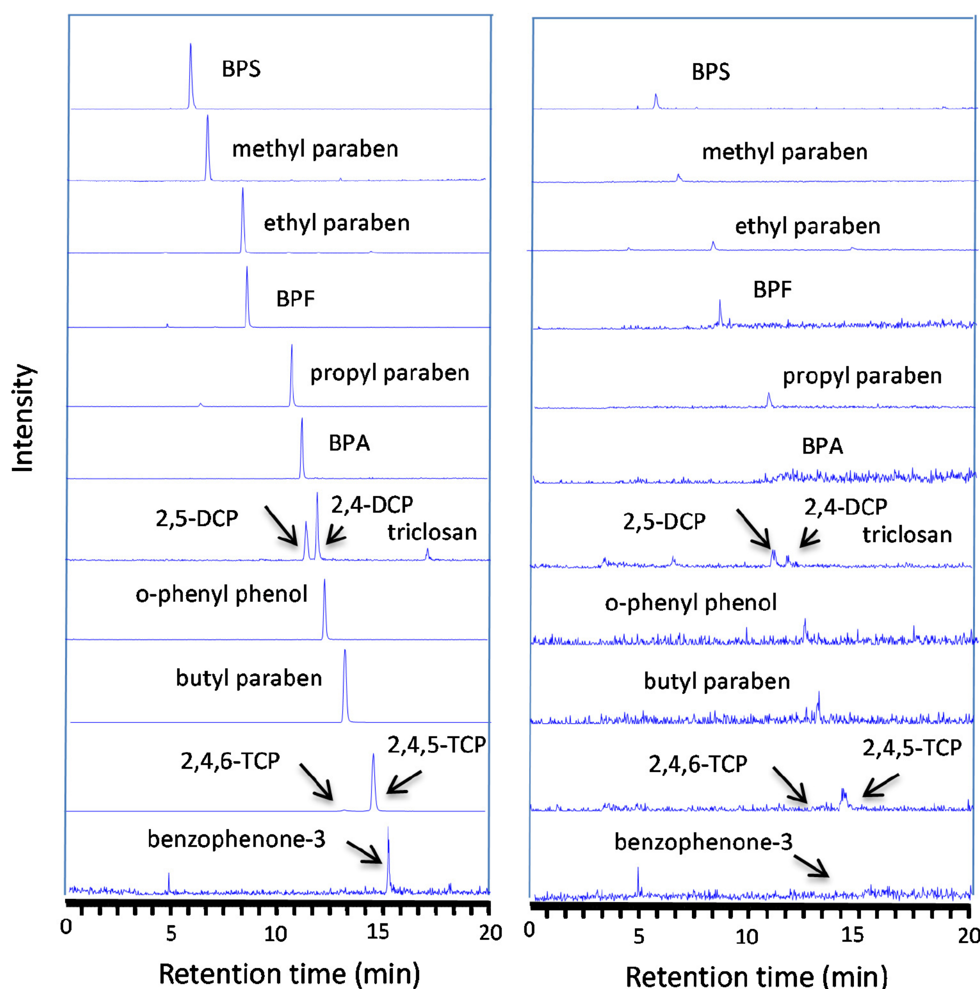

Fig. 1. Typical HPLC–MS/MS extracted ion chromatograms from standard at concentration of 1 ng/mL[a] (left) and blank urine (right). *Note*: DCP and TCP indicate dichlorophenol and trichlorophenol, respectively. [a]The *y*-axis (intensity) was plotted normalized to the largest peak due to the much different sensitivities from the different analytes.

Table 2
Median concentrations (ng/mL), concentration ranges, and frequency of detection of BPA, BPF and BPS in 100 urine samples.[a,b]

Urine (*N* = 100)	Total BPA (free BPA)	Total BPF (free BPF)	Total BPS (free BPS)
Median Conc. (ng/mL)	0.72	0.08	0.13
	(<LOD)	(<LOD)	(<LOD)
Conc. Range (ng/mL)	<LOD-37.7	<LOD-212	<LOD-12.3
	(<LOD-0.11)	(<LOD-0.87)	(<LOD-0.14)
Detection frequency (%)	95	55	78
	(1)	(5)	(5)
Mean conjugate (%)[c]	100	100	97

[a]Concentrations <LOD (0.03 ng/mL, BPS; 0.06 ng/mL, BPF; 0.1 ng/mL, BPA) were imputed a value of LOD divided by the square root of 2 for the statistical calculations [18].

[b] Urine samples were collected in Atlanta, GA during 2009–2012 at different time throughout the day from a diverse group of male and female adult volunteers with no documented occupational exposure to the target phenols.

[c] To calculate the mean percent of the conjugated species, we included only samples with detectable total concentrations.

the bisphenols or degradation of their conjugates during sampling, storing or handling of the urine.

4. Conclusions

We developed a sensitive on-line SPE–HPLC–MS/MS method with peak focusing for the concurrent quantification of BPA, BPF, BPS and 11 other phenols in human urine with minimal sample pretreatment. Compared with other methods developed for measuring BPA and BPS in urine, the current method is fully automated and requires a smaller amount of sample (100 μL). Furthermore, it allows for the simultaneous quantification of BPF and of 11 other phenols with a better sensitivity than our previously published methods. The current method is rugged as well as labor and cost-effective. Our preliminary data in a convenience sample of US adults also suggest that detection frequency and urinary concentrations of BPF and BPS are lower than those of BPA. The current method allows for the quick analysis of large number of samples for epidemiologic studies to evaluate the human exposure to these compounds.

Disclaimer

The use of trade names is for identification only and does not constitute endorsement by the US Department of Health and Human Services or the Centers for Disease Control and Prevention.

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jchromb.2013.11.009.

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