



Full length article

## Glucuronide and sulfate conjugates of tetrabromobisphenol A (TBBPA): Chemical synthesis and correlation between their urinary levels and plasma TBBPA content in voluntary human donors



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### ARTICLE INFO

#### Article history:

Received 27 June 2016

Received in revised form 16 September 2016

Accepted 22 September 2016

Available online 4 October 2016

#### Keywords:

Tetrabromobisphenol-A

Glucuronide and sulfate conjugates

Body fluids

Human plasma

Human urine

### ABSTRACT

3,3',5,5'-Tetrabromobisphenol-A (TBBPA) is an important brominated flame retardant in epoxy, vinyl esters and polycarbonate resins. Previous studies have already shown the occurrence of its Phase II metabolites, TBBPA-glucuronide and sulfate conjugates, in human urine, after oral administration of TBBPA. The main objective of this work is to examine correlations among level of TBBPA in human blood and those of its Phase II metabolites in human urine. Four water-soluble TBBPA conjugates were synthesized, purified and characterized. An analytical protocol using solid-phase extraction and liquid chromatography–electrospray tandem mass spectrometry (SPE-LC-MS/MS) quantification was developed for the simultaneous analysis of these glucuronide and sulfate conjugates in human urine samples. TBBPA and its Phase II metabolites in paired human plasma and urine samples collected randomly from 140 voluntary donors in Hong Kong SAR, China, were determined. One or more TBBPA conjugates were detected in all of the urine samples, with concentration ranging from 0.19 to 127.24  $\mu\text{g g}^{-1}$ -creatinine. TBBPA was also quantified in >85% of the plasma and urine samples. Strong correlations were observed between TBBPA content in plasma and the total amount of TBBPA-related compounds in urine.

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### 1. Introduction

Environmental endocrine disrupting chemicals (EDCs) are those chemical species that occur in the various environmental compartments that can affect normal hormonal signaling in living organisms (Nicolopoulou-Stamati et al., 2001). Their potential risks to environmental and human health have aroused much public concerns (Norris and Carr, 2006). 3,3',5,5'-Tetrabromobisphenol-A (TBBPA) is a synthetic chemical widely used as a brominated flame retardant, and is, therefore, commonly found in a wide variety of daily appliances (Birnbaum and Staskal, 2004; Shi et al., 2009a). Recent studies have revealed its endocrine disrupting impacts on the reproduction and the functioning of thyroid hormones of laboratory animals (Saegusa et al., 2009; van der

Ven et al., 2008). Toxicological and human exposure data that we have gathered thus far warrant our concern that TBBPA may have already exerting their adverse impacts on human health (Kim and Oh, 2014). As Southern China is one of the major manufacturing hubs for consumer products in the world, there is much concern about environmental contamination and public health risk posed by EDCs in the region (Feng et al., 2012; He et al., 2010; Shi et al., 2009a; Tan et al., 2016; Tang et al., 2015; Wang et al., 2015).

At the moment, the most commonly adopted approach for the estimation of population exposure to selected environmental contaminants is the direct quantification of their levels in human tissues, mainly whole blood/serum (Cariou et al., 2008; Dirtu et al., 2008; Dirtu et al., 2010; Fujii et al., 2014a; Fujii et al., 2014b; Jakobsson et al., 2002; Kim and Oh, 2014; Nagayama et al., 2000; Shi et al., 2013b; Thomsen et al., 2001; Thomsen et al., 2002), and breast milk (Abdallah and Harrad, 2011; Carignan et al., 2012; Cariou et al., 2008; Fujii et al., 2014b; Kang et al., 2015; Lankova et al., 2013; Nakao et al., 2015; Shi et al., 2009b; Shi et al., 2013a, 2013b) from voluntary donors recruited from the population. Other less commonly used tissues for exposure estimation include adipose tissue (Cariou et al., 2008; Johnson-Restrepo et al.,

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2008), and hair (Pellizzari et al., 1978). Nevertheless, sampling human tissues for contaminant analysis is intrusive and difficult to achieve on a large scale, especially for healthy subjects who are not hospitalized or required to go through clinical diagnostic tests. Also, this approach can only provide a snapshot view of the exposure situation, unless a long-term and extensive sampling arrangement is available, which demands huge amount of resources to sustain. Sampling of breast milk can be considered a non-invasive operation, but is very much that restricted to lactating women within a relatively narrow age distribution (Landrigan et al., 2002).

Because of their high molecular mass, excretion via urine only constitutes a minor elimination route for TBBPA and its metabolites in human (Szymańska et al., 2001, Kuester et al., 2007). Nevertheless, glucuronide and sulfate conjugated Phase II metabolites of TBBPA have been quantified in human urine (Fini et al., 2012, Schauer et al., 2006). In vitro studies on the biotransformation of TBBPA carried out on rat hepatocytes have also revealed the production of TBBPA sulfate and glucuronide conjugates (Nakagawa et al., 2007). Thus, it is deemed worthy of examining correlations of these glucuronide and sulfate conjugates of TBBPA in human urine with TBBPA exposure. In this context, we report the synthesis and purification of all the four common Phase II metabolites of TBBPA (TBBPA mono-/di-glucuronide and mono-/di-sulfate conjugates), and their use as authentic standards for the development of an analytical protocol for their determination in human urine. We have also studied the stability of these TBBPA Phase II metabolites in human urine and derived means to preserve them for quantitative determination. Finally, correlations among their levels in human urine and that of TBBPA in human blood are investigated.

## 2. Materials and methods

### 2.1. Safety precautions

Extra precaution was practiced in the handling of human blood and urine samples. Double latex gloves, facemasks and eye-protection goggles were worn all the time during their handling, spiking and transferal. All the spent samples after analysis were collected in a separated close-lipped container with proper clinical waste labels. These spent samples and all the used personal protection items were treated as clinical wastes and were collected and disposed of in accordance with the “Code of Practice for the Management of Clinical Waste” issued by the Environmental Protection Department of the Hong Kong SAR Government.

### 2.2. Sample collection

Human studies were performed in accordance with the guidelines and approval of the research ethics committee of City University of Hong Kong. Parallel human plasma and urine samples from 140 voluntary donors were collected from April to August 2012, by registered doctors and nurse, at Queens Mary Hospital, Hong Kong SAR. Of the 140 donors, 66 were male and 74 were female. The age range of these volunteers was from 18 to 96 year-old, with the mean age =  $47.1 \pm 18.2$  year-old. These volunteers were subdivided into different age groups for comparison as following: age 18 to 25 ( $n = 17$ ); age 26 to 35 ( $n = 18$ ); age 36 to 45 ( $n = 44$ ); age 46 to 55 ( $n = 24$ ); age 56 to 65 ( $n = 15$ ); age 66 to 80 ( $n = 13$ ) and age > 80 ( $n = 9$ ).

Whole blood samples were collected using the standard phlebotomy technique in vacutainer tubes containing sodium heparin anticoagulant (Vacuette, Greiner bio-one, GmbH, Austria). The blood was then centrifuged at  $1500 \times g$  for 25 min. Plasma was removed from the top of the tube. Urine samples were collected in 100 mL sterilized glass bottles and stored at  $-80^\circ\text{C}$ , within 15 min after sampling, until being analyzed. Urine sample from each donor was subdivided into three replicate samples before low temperature storage. All samples were carefully labeled and documented. Upon analysis, samples were

thawed, and 10 mL of each sample was taken for creatinine content determination. Creatinine determination was carried out by a kinetic colorimetric assay based on the modified Jaffe method (Bonsnes and Tausky, 1945) using the Roche Modular System (Roche Diagnostics, IN, USA), with an analytical range between 360 and  $57,500 \text{ mmol L}^{-1}$ .

### 2.3. Synthesis, purification and characterization of TBBPA glucuronides and sulfate conjugates

The general synthetic routes for the TBBPA glucuronide and sulfate conjugates are outlined in Scheme S1 of the Supporting information. Detail synthetic and purification procedures and characterization data are also given in the Supporting information.

### 2.4. Sample extraction and cleanup

#### 2.4.1. TBBPA in human plasma

Literature method (Dirtu et al., 2008, Hovander et al., 2000), with slight modifications, was adopted for the extraction of TBBPA in human plasma.  $^{13}\text{C}_{12}$ -TBBPA (1 ng) was spiked to 1 mL of human plasma sample. The spiked plasma sample was allowed to stand at room temperature for 10 min before 50  $\mu\text{L}$  of concentrated hydrochloric acid (37%), Milli-Q water (2 mL) and isopropanol (3 mL) were added. The mixture was then extracted by hexane/MTBE ( $3 \times 5 \text{ mL}$ , 1:1, v/v). The organic fractions were combined and partitioned with a 1% KCl solution (3 mL) followed by evaporation to dryness under a gentle stream of nitrogen. Lipid content of the plasma sample was determined gravimetrically. The residue was re-dissolved in hexane (4 mL) and partition with potassium hydroxide (2 mL, 0.5 M in 50% ethanol) to ionize the phenolic analytes. Neutral compounds were separated by hexane ( $2 \times 4 \text{ mL}$ ). The aqueous layer was acidified by hydrochloric acid (2 mL, 0.5 M), then the phenolic compounds were extracted by hexane/MTBE ( $2 \times 4 \text{ mL}$ , 9:1, v/v). Phenolic fraction was evaporated to dryness under a gentle stream of nitrogen and reconstituted in 1 mL of 5% acetone in hexane. The mixture was subjected to a SPE clean-up using a Sep.-Pak Florisil cartridge previously conditioned by DCM/MeOH (6 mL, 4:1, v/v) and 5% acetone in hexane (6 mL) at the flow rate of 1 drop/s. The cartridge then washed by 5% acetone in hexane (6 mL). The cartridge was the dried under reduced pressure and the BPA and BPA- $d_{16}$  were eluted by DCM/MeOH (10 mL, 4:1, v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen. Analytes were reconstituted in 50  $\mu\text{L}$  isooctane containing 5 ng pyrene- $d_{10}$  as an internal standard and the phenolic analytes were derivatized by 50  $\mu\text{L}$  BSTFA with 1% TMCS at  $70^\circ\text{C}$  for an hour.

#### 2.4.2. TBBPA in human urine

Literature method (Ho et al., 2016), with slight modifications, was adopted from the extraction of TBBPA from human urine. Briefly,  $^{13}\text{C}_{12}$ -TBBPA (1 ng) was spiked in 5 mL of human urine. The spiked sample was then allowed to stand at room temperature for 10 min. Then, 50  $\mu\text{L}$  of formic acid was added, followed by extraction with ethyl acetate ( $3 \times 5 \text{ mL}$ ). The organic fractions were combined and evaporated to dryness under a gentle stream of nitrogen. This was followed by reconstitution in 5% acetone in hexane (1 mL), and SPE clean-up with a Sep.-Pak Florisil cartridge previously conditioned by DCM/MeOH (6 mL, 4:1, v/v) and 5% acetone in hexane (6 mL) at a rate of 1 drop/s. The cartridge was then eluted by 5% acetone in hexane (6 mL). The cartridge was the dried under reduced pressure and the TBBPA and  $^{13}\text{C}_{12}$ -TBBPA were eluted by DCM/MeOH (10 mL, 4:1, v/v). The eluate was evaporated to dryness under a gentle stream of nitrogen. Analytes were reconstituted in 50  $\mu\text{L}$  isooctane containing 5 ng pyrene- $d_{10}$  as an internal standard and the phenolic analytes were derivatized by 50  $\mu\text{L}$  BSTFA with 1% TMCS at  $70^\circ\text{C}$  for an hour.

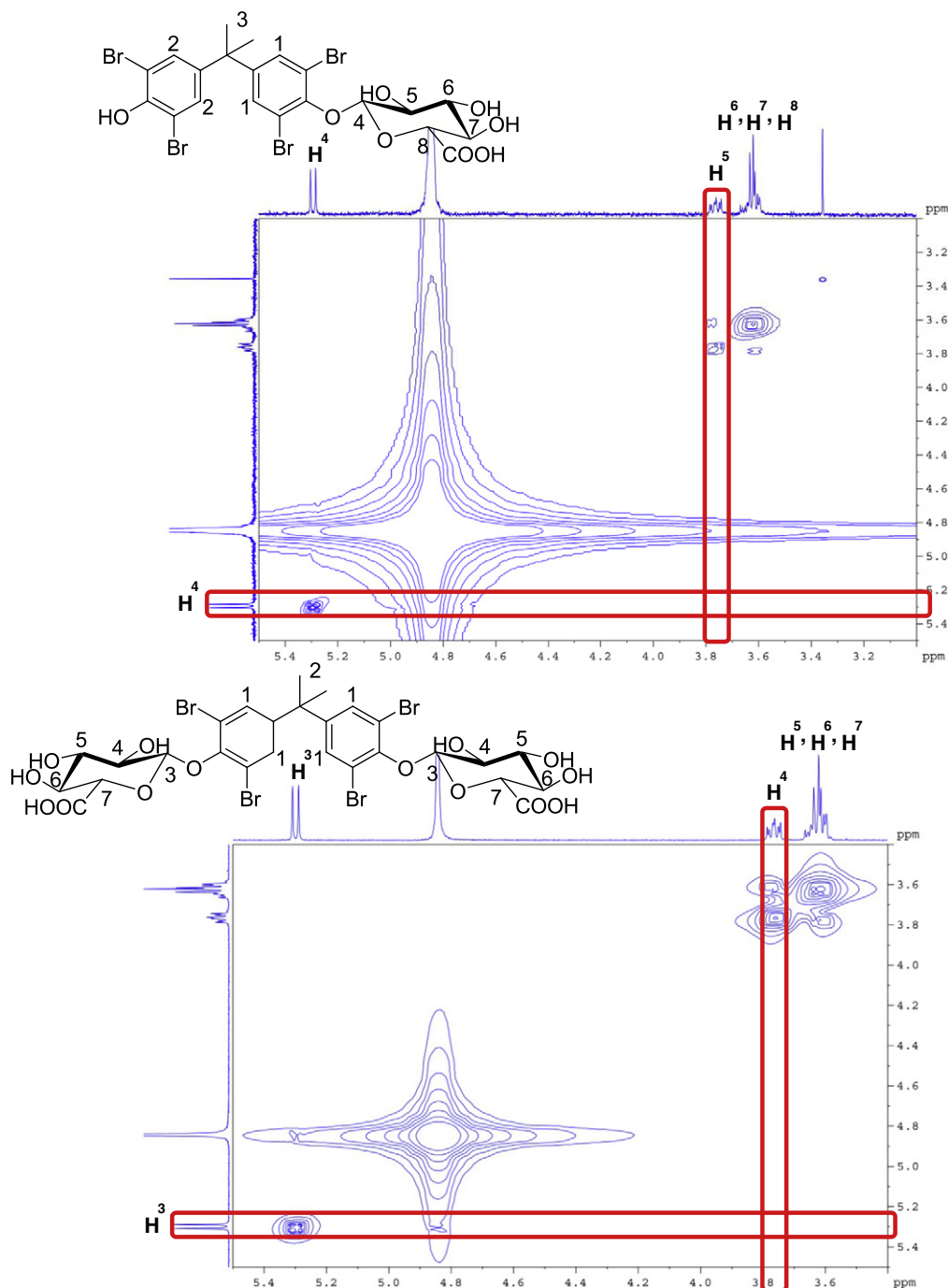
### 2.4.3. TBBPA glucuronide and sulfate conjugates in human urine

Literature method (Ho et al., 2016), with slight modifications, was adopted from the extraction of the highly water-soluble TBBPA conjugates from human urine.  $^{13}\text{C}_{12}$ -TBBPA (10 ng) was spiked in 5 mL of human urine. The spiked sample was then allowed to stand at room temperature for 10 min. Then, was mixed with 2 M sodium acetate buffer (5 mL, pH = 5) and then loaded at the flow rate of 1 drop/s onto an Oasis® WAX cartridge previously conditioned with 5 mL of methanol, then 5 mL of Milli-Q water, then 5 mL of 2 M sodium acetate buffer. The cartridge was then washed by a 2 M sodium acetate buffer (5 mL, pH = 5), Milli-Q water (5 mL), and 2% acetic acid in methanol (5 mL). The sorbent was then dried under reduced pressure and the

glucuronide and sulfate conjugates were eluted by 5%  $\text{NH}_4\text{OH}$  in methanol (10 mL). The elute was evaporated to dryness under a gentle stream of nitrogen at 50 °C and was then reconstituted in a mixture of acetonitrile and water (1:1, 100  $\mu\text{L}$ ) containing  $^{13}\text{C}_{12}$ -tetrachlorobisphenol A ( $^{13}\text{C}_{12}$ -TCBPA, 50 ng). The sample was finally analyzed by HPLC-MS/MS.

### 2.5. Quality control (QC), quality assurance (QA) and statistical analysis

Details on QA/QC and statistical analysis for this study are given in the Supporting Information.



**Fig. 1.**  $^1\text{H}$ - $^1\text{H}$  ROESY 2D-NMR spectra of TBBPA mono-glucuronide (upper) and TBBPA di-glucuronide (lower). The absence of cross signal between the C1 and C2 carbons of the glucuronide rings indicates the  $\beta$ -conformation of the rings.

### 3. Results and discussion

#### 3.1. Synthesis and purification of the glucuronide and sulfate conjugates of TBBPA

Mono- and di-sulfate conjugate of TBBPA were prepared according to the previous published method with few modifications (Burkhardt and Lapworth, 1926). The excess chlorosulfonic acid cannot be neutralized by inorganic strong base like KOH or  $K_2CO_3$  because the sulfate ester linkage will be cleaved in the presence of inorganic bases. Instead, the products have to be precipitated by triethylamine and subjected to purification by HPLC. The sulfate conjugates were in their triethylamine salts. D-(+)-glucuronic acid  $\gamma$ -lactone was protected by acetic anhydride in the presence of perchloric acid and then the activated C1 position was brominated by HBr (33%) in glacial acetic acid. The sugar moiety was then linked to TBBPA in an appropriate mole ratio to obtain TBBPA mono-2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranosiduronic acid methyl ester and TBBPA di-2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranosiduronic acid methyl ester. The protected sugar moieties were then deprotected by excess KOH in methanol to give the desire products. Such a synthetic route is different from that adopted in our previous study for the synthesis of bromophenol glucuronides (Ho et al., 2012). In fact, we have tried that synthetic route, but observe a cleavage of the ether linkages between TBBPA and the sugar moieties under TEMPO mediated oxidation of primary alcohols. Stereochemistry of our synthesized TBBPA mono-/di glucuronides was confirmed by  $^1H$ - $^1H$  ROESY 2D-NMR (Fig. 1) to sustain the  $\beta$ -confirmation, which is the same as natural glucuronide metabolites in human (Mulder, 1992).

#### 3.2. Validation of analytical methods

Previous studies on the pharmacokinetics and toxicokinetics of TBBPA in human and mammalian animal models have already reported the occurrence of urinary TBBPA glucuronide and sulfate conjugates (Fini et al., 2012, Hakk et al., 2000, Nakagawa et al., 2007, Knudsen et al., 2014, Riu et al., 2011, Schauer et al., 2006, Zalko et al., 2006). Yet, those Phase II metabolites of TBBPA have only been qualitatively identified. To the best of our knowledge, no information is available on their relative abundance in human urine, not to mention any correlation with their parent TBBPA compound in human blood. Thus, we set out to develop an analytical protocol for the qualitative and quantitative determination of TBBPA and its Phase II metabolites in human urine. Table 1 tabulates all the Multiple Reaction Monitoring (MRM) transitions adopted for the identification and quantification of TBBPA, and TBBPA glucuronide and sulfate conjugates in this study. Good linear LC-MS/MS responses were obtained for different concentrations of mono-/di-glucuronide and sulfate substituted conjugates of TBBPA covered the concentration range of 0.5 to 1000 ng mL<sup>-1</sup>. The correlation coefficients of the best-fitted line,  $r^2$ , were in the range of 0.9987 to 0.9997 for the four different substituted glucuronide and sulfate conjugates of TBBPA. In addition, linearity of GC-MS/MS responses to parent TBBPA and  $^{13}C_{12}$ -TBBPA was analyzed for the range from 0.5 to 1000 ng mL<sup>-1</sup>. The correlation coefficients,  $r^2$ , of the best-fitted lines are 0.9993 and 0.9994 for TBBPA and  $^{13}C_{12}$ -TBBPA respectively. Repeatability and analyte recovery were evaluated by the consecutive analysis of seven independent artificial urine samples spiked with 0.5 ng mL<sup>-1</sup> of each of the conjugates. Table 2 summaries the optimize performance of the analytical protocol for the determination of the TBBPA Phase II conjugates. The method detection limits (MDLs) for all the conjugates were  $\leq 6.4$  ng g<sup>-1</sup> creatinine. Finally, robustness of the analytical protocol was assessed by analyzing the same spiked sample once a week for seven weeks. No variation in the relative retention time (RRT) of the conjugates whatsoever was observed. Fluctuations in the corresponding chromatographic peak areas of the conjugates were within 5%.

**Table 1**

LC-MS/MS transitions and MS parameters of TBBPA Phase II conjugates (upper) and GC-MS/MS transitions and MS parameters of TBBPA analysis (lower) adopted in this study.

Analytes	MRM Transitions (m/z)	Collision Energy (V)			
TBBPA Di-TMS derivatives	673 → 73	34			
	673 → 365	10			
$^{13}C_{12}$ -TBBPA Di-TMS derivatives (surrogate standard)	685 → 73	34			
	685 → 371	10			
Pyrene- $d_{10}$ (internal stranded)	212 → 208	36			
Analytes	MRM transitions (m/z)	Declustering potential (V)	Entrance potential (V)	Collision energy (V)	Collision cell potential (V)
TBBPA mono-glucuronide	719 → 175	-53	-2.5	-27	-1.5
	719 → 113	-55	-3	-45	-0.5
	359 → 79	-17	-2	-60	-0.5
TBBPA di-glucuronide	895 → 113	-73	-3	-50	-0.5
	447 → 85	-30	-2	-35	-1
	447 → 59	-30	-1.5	-50	-0.5
TBBPA mono-sulfate	623 → 543	-50	-4.5	-35	-2.5
	623 → 79	-55	-4	-100	-1
TBBPA di-sulfate	311 → 311	-35	-1.5	-65	-1
	351 → 311	-30	-4	-23	-3
$^{13}C_{12}$ -TBBPA (internal standard)	351 → 79	-30	-4	-6	-1

#### 3.3. Stability of TBBPA glucuronide and sulfate conjugate in human urine

Stability of the synthesized TBBPA Phase II metabolites under various preservation conditions was assessed, and results are shown in Fig. 2. Similar to those Phase II metabolites of bromophenols and bisphenol-A, the glucuronide and sulfate conjugates of TBBPA are rather stable at low temperature (Ho et al., 2012, Ho et al., 2016). They remain unchanged in 2 weeks and have only decreased about one fifth in 30 days. On the other hand, neither addition of formaldehyde, nor sodium azide, was able to suppress their rather rapid degradation. Thus, low temperature storage ( $-80$  °C) was adopted to preserve human urine samples in this study.

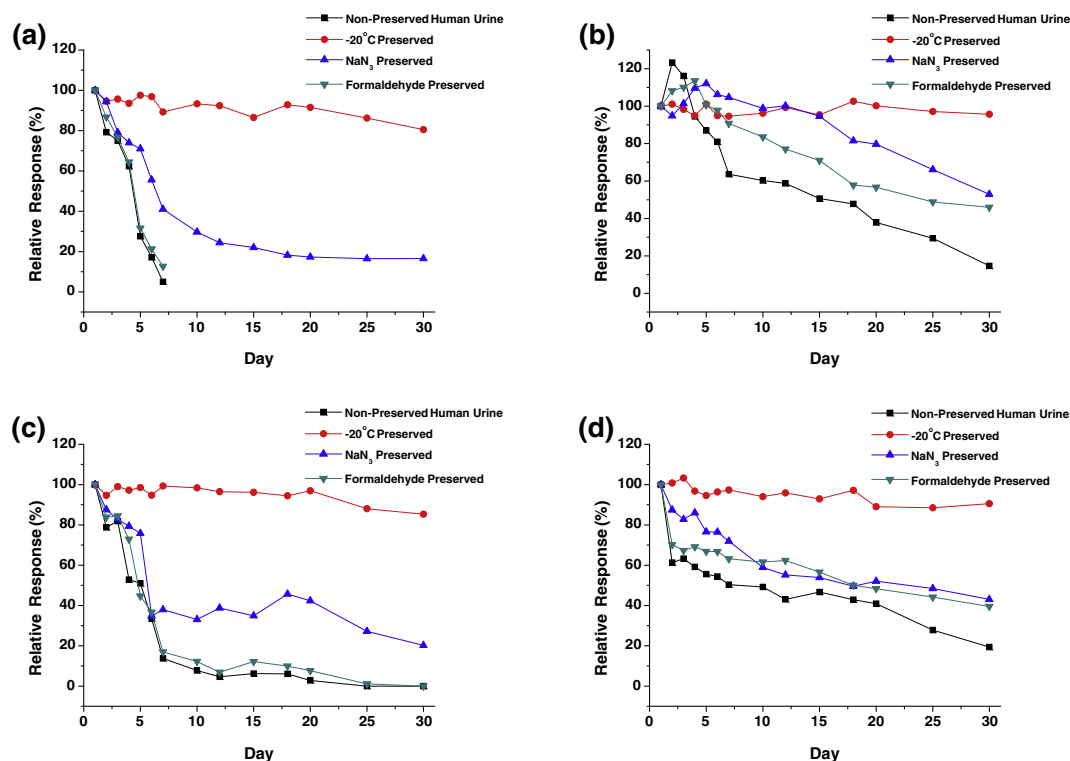
#### 3.4. Levels of TBBPA in human plasma and human urine samples

TBBPA was quantified in ca. 93% of the plasma samples of the 140 voluntary donors. The geometric mean content was 3.65 ng g<sup>-1</sup> l.w.,

**Table 2**

Performance of the SPE-LC-MS/MS analytical protocol for the determination of the TBBPA glucuronide and sulfate conjugates, and the SPE-GC-MS/MS analytical protocol for the determination of the TBBPA in artificial urine matrix.

TBBPA/TBBPA conjugates	Relative repeatability (%)	Inter-day reproducibility (%)	Recovery (%)	MDL (ng g <sup>-1</sup> creatinine)
SPE-LC-MS/MS				
TBBPA mono-glucuronide	4.8	8.1	96.8	5.5
TBBPA mono-sulfate	3.3	5.3	106.2	2.2
TBBPA di-glucuronide	4.9	8.0	87.4	6.4
TBBPA di-sulfate	4.9	7.9	76.4	2.2
SPE-GC-MS/MS				
TBBPA	2.1	3.4	85.2	13.3
$^{13}C_{12}$ -TBBPA	3.6	4.0	81.1	17.3



**Fig. 2.** Stability of TBBPA conjugates under chemical and physical means of preservation: (a) TBBPA mono-glucuronide, (b) TBBPA mono-sulfate, (c) TBBPA di-glucuronide, and (d) TBBPA di-sulfate.

(95% confidence interval: 3.44–3.88 ng g<sup>-1</sup> l.w). This plasma concentration of TBBPA is quite comparable to that revealed in other studies on the general population of China and Japan (Nagayama et al., 2000, Shi et al., 2013b), and workers (Jakobsson et al., 2002, Thomsen et al., 2001). On the other hand, it is higher than those in the general population of European countries (Jakobsson et al., 2002, Thomsen et al., 2002), except the maternal serum and cord serum of French mothers (Cariou et al., 2008), and plasma samples collected in Belgium (Dirtu et al., 2008). High concentration of TBBPA has also been detected in Korean infant-mother paired serum samples (Kim and Oh, 2014). Such a level of TBBPA in human plasma collected in Hong Kong SAR is probably due to the high production and consumption rate of TBBPA in the Pearl River Delta region of Southern China. Previous studies in Southern China

have already detected high TBBPA content in bird samples collected near e-waste recycling sites (He et al., 2010). There is no obvious difference in the plasma TBBPA content between male and female donors (natural-logarithmic transformed,  $P = 0.248$ ). Also, there is no significant difference in the median level of TBBPA in human plasma among age groups (natural-logarithmic transformed,  $P = 0.947$ ). TBBPA was also quantified in >85% of urine samples of the voluntary donors. Its geometric mean level was 0.19  $\mu\text{g g}^{-1}$  creatinine (95% confidence interval: 0.13–0.29  $\mu\text{g g}^{-1}$  creatinine) (Table 3). No statistically significant difference is observed between male and female donors (natural-logarithmic transformed,  $P = 0.368$ ). There is also no statistical difference in the urinary TBBPA content among age groups (natural-logarithmic transformed,  $P = 0.109$ ).

**Table 3**  
Concentrations of TBBPA and TBBPA mono-/di-glucuronide and sulfate conjugates in paired human plasma (ng mL<sup>-1</sup>) and human urine samples ( $\mu\text{g g}^{-1}$  creatinine) from Hong Kong, South China (n = 140).

	Total (n = 140)			Male (n = 66)			Female (n = 74)		
	GM <sup>a</sup> (95% CI) <sup>b</sup>	Min-max	% of detection	GM <sup>a</sup> (95% CI) <sup>b</sup>	Min-max	% of detection	GM <sup>a</sup> (95% CI) <sup>b</sup>	Min-max	% of detection
Human plasma samples									
TBBPA	3.65 (3.44–3.88)	N.D. – 7.49	92.9	3.55 (3.25–3.88)	N.D. – 7.21	90.9	3.82 (3.51–4.16)	N.D. – 7.49	94.6
Human urine samples									
TBBPA	0.19 (0.13–0.29)	N.D. – 88.41	88.6	0.14 (0.08–0.25)	N.D. – 15.73	93.9	0.23 (0.13–0.44)	N.D. – 88.41	85.1
TBBPA mono-glucuronide	1.69 (1.20–2.38)	N.D. – 118.06	86.4	1.24 (0.80–1.21)	N.D. – 118.06	87.9	2.24 (1.34–3.75)	N.D. – 103.23	85.1
TBBPA di-glucuronide	1.74 (1.33–2.26)	N.D. – 95.57	99.3	1.32 (0.87–2.01)	N.D. – 22.09	98.5	2.21 (1.60–3.06)	N.D. – 95.57	100
TBBPA mono-sulfate	0.63 (0.51–0.78)	N.D. – 26.86	82.1	0.48 (0.36–0.64)	N.D. – 11.04	81.8	0.81 (0.61–1.07)	N.D. – 26.86	82.4
TBBPA di-sulfate	0.89 (0.69–1.14)	N.D. – 25.80	82.9	0.81 (0.55–1.21)	N.D. – 25.80	84.8	0.96 (0.70–1.32)	N.D. – 8.29	81.1

N.D.: not detected.

<sup>a</sup> GM: geometric mean.

<sup>b</sup> CI: confidence interval.

**Table 4**  
Correlation coefficients between parent TBBPA in human plasma and its Phase II metabolites in urine.

	Total (n = 140)		Male (n = 66)		Female (n = 74)	
	r	P	r	P	r	P
Urinary content (in terms of mass concentration)						
ln[parent TBBPA]	0.547	<0.05	0.534	<0.05	0.586	<0.05
ln[ $\Sigma$ TBBPA Phase II metabolites]	0.626	<0.05	0.668	<0.05	0.599	<0.05
ln[{parent TBBPA} + $\Sigma$ {TBBPA Phase II metabolites}]	0.886	<0.005	0.873	<0.05	0.897	<0.05
Urinary content (in terms of molar concentration)						
ln[TBBPA-related compounds]	0.913	<0.005	0.901	<0.05	0.927	<0.05

### 3.5. Levels of TBBPA glucuronide and sulfate conjugates in human urine samples

Concentrations of TBBPA glucuronide and sulfate conjugates in human urine samples are summarized in Table 3. At least one of four TBBPA Phase II metabolites was detected in the urine samples of the voluntary donors. TBBPA di-glucuronide was the most frequently detected. Levels of urinary TBBPA mono-glucuronide and di-glucuronide are comparable, with geometric means of  $1.69 \mu\text{g g}^{-1}$  creatinine (95% confidence interval: 1.20–2.38) and  $1.74 \mu\text{g g}^{-1}$  creatinine (95% confidence interval: 1.33–2.66  $\mu\text{g g}^{-1}$  creatinine), respectively, which are much higher than those of TBBPA mono- & di-sulfate. Of the four TBBPA conjugates, only the urinary TBBPA di-glucuronide showed statistically difference in concentration between male and female (natural-logarithmic transformed,  $P = 0.012$ ). Higher concentration of TBBPA di-glucuronide was found in women (median =  $0.883 \mu\text{g g}^{-1}$  creatinine) compared to men (median =  $0.368 \mu\text{g g}^{-1}$  creatinine). However, in term of age, there is no statistically difference observed for all the TBBPA Phase II conjugates.

### 3.6. Correlation studies

We employed Pearsons Product Moment to examine relationships among urinary levels (natural-logarithmic transformed) of TBBPA and its conjugates and that of TBBPA in the corresponding blood plasma of voluntary donors. Results are tabulated in Table 4 and shown in Figs. 3 and 4. In terms of mass concentration of TBBPA, only moderate correlation,  $r = 0.547$ , is found between blood plasma and urine. Correlation between the mass concentration of plasma TBBPA and that of all the quantified TBBPA Phase II conjugates in urine is also not very substantial ( $r = 0.626$ ). Indeed, a much improved correlation,  $r = 0.886$ , is found between the mass concentration of plasma TBBPA and that of urinary “TBBPA-related compounds” (i.e. the parent TBBPA and all the TBBPA glucuronide and sulfate conjugates).

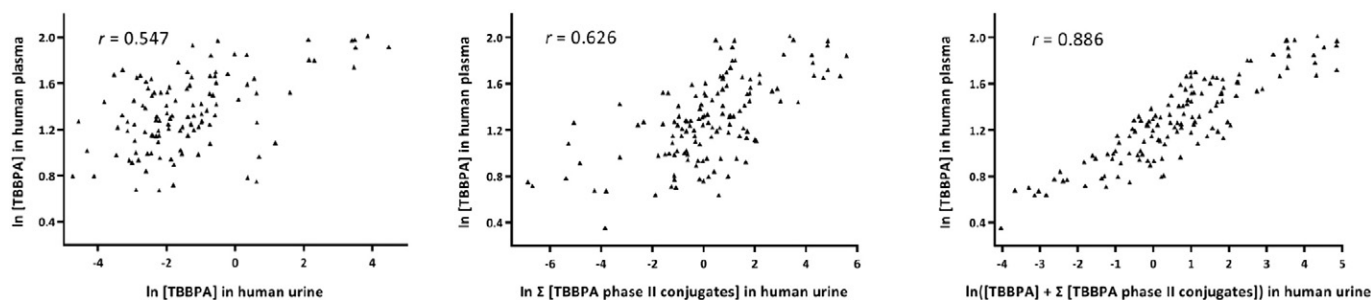
Expressing the content of urinary metabolites of organic contaminants in terms of their mass concentration may not be able to truly reflect the quantity that had been transformed from their parent compounds as the resulting conjugation groups affect their molecular masses. A survey of environmental exposure of pregnant women to bisphenol A (BPA), Gerona et al. (2016) converted the mass concentrations of BPA mono-glucuronide and sulfate conjugates into their “BPA-equivalent” mass concentrations using the ratios of the molecular mass of BPA to that of its corresponding conjugates as the weighing factors. Thus, to further explore the correlation between TBBPA in human plasma and its metabolites in human urine, we calculated the molar concentration (i.e. in  $\text{mol mL}^{-1}$  for plasma content and  $\text{mol g}^{-1}$ -creatinine for urinary content) of TBBPA and its Phase II conjugates in the plasma and urine samples (Eq. 1):

$$\text{Molar concentration of contaminant} = \frac{\text{mass concentration of contaminant}}{\text{molecular mass of contaminant}} \quad (1)$$

Molecular mass of TBBPA ( $543.9 \text{ g mol}^{-1}$ ), TBBPA mono-glucuronide ( $703.0 \text{ g mol}^{-1}$ ), TBBPA di-glucuronide ( $894.1 \text{ g mol}^{-1}$ ), TBBPA mono-sulfate (triethylammonium salt) ( $725.1 \text{ g mol}^{-1}$ ) and TBBPA di-sulfate (bis-triethylammonium salt) ( $906.4 \text{ g mol}^{-1}$ ) were used to transform the mass concentration of TBBPA and its glucuronide and sulfate conjugates into their corresponding molar concentration in the plasma and urine samples. Re-examining the relationship between plasma TBBPA content and that of all “TBBPA-related compounds” in urine reveals an even stronger correlation with  $r = 0.913$  (Fig. 4).

Previous studies have revealed that TBBPA is efficiently metabolized in the liver by glucuronyl- and sulfotransferases, which are, in turn, eliminated mainly into bile (Schauer et al., 2006, Kuester et al., 2007). Because of the resulting enterohepatic circulation, systemic bioavailability of TBBPA in mammalian models, including human, is not high. Half-life of TBBPA in human plasma is approximately 2 days (Hagmar et al., 2000, Schauer et al., 2006). This may explain the relatively poor association between the plasma TBBPA content and the urinary levels of its Phase II conjugates. Nevertheless, this study reveals that the combined content of TBBPA and all of its Phase II conjugates shows much improved correlation with the plasma TBBPA level. In view of the relatively complex metabolism and elimination pathways of TBBPA in human, it is deemed too early to judge whether such a urinary content of “TBBPA-related compounds” can be a reliable population exposure marker for TBBPA. Further works to study their relationships with TBBPA in other body fluids, e.g. bile, and tissues are definitely needed.

In conclusion, all the four common Phase II metabolites of TBBPA have been successfully synthesized, purified and characterized as authentic standards for analytical purposes. An SPE-LC-MS/MS analytical protocol has been developed for their quantitative determination in human urine. Through the analysis of paired blood plasma and urine samples from 140 voluntary donors in Hong Kong SAR, China, a good



**Fig. 3.** Correlation of plasma level of TBBPA with: (i) TBBPA content in human urine (left); (ii) total content of all the four Phase II conjugates of TBBPA in human urine (middle); (iii) total content of “TBBPA-related compounds” (i.e. both the parent TBBPA and its Phase II conjugates) in human urine (right). Levels of TBBPA and its Phase II conjugates have been natural-logarithmic transformed.

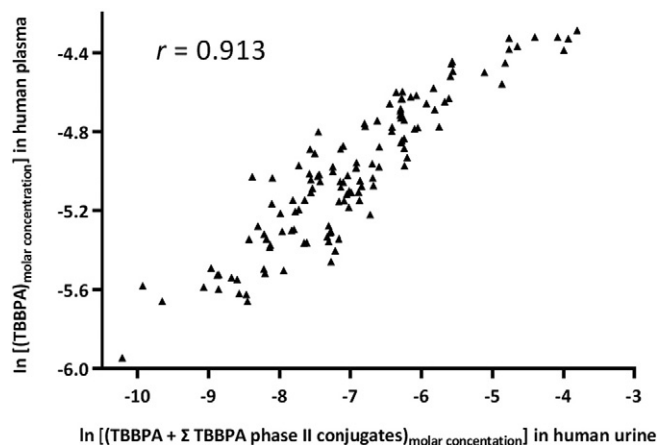


Fig. 4. Correlation of molar concentrations (natural-logarithmic transformed) TBBPA in plasma with that of the “TBBPA-related compounds” in urine of voluntary donors.

correlation has been revealed between plasma level of TBBPA and the urinary content of all the “TBBPA-related compounds” (parent TBBPA and all of its four Phase II conjugates). We hope that these results are useful for the establishment of more convenient population exposure markers for TBBPA.

#### Acknowledgement

This work is supported by the State Key Laboratory for Marine Pollution, City University of Hong Kong, and a GRF grant CityU 160513 from the Research Grants Council of the Hong Kong Special Administrative Region, China. Prof. Giesy was supported by the program of 2012 “Great Concentration Foreign Experts” (#GDW20123200120) funded by the State Administration of Foreign Experts Affairs, the P.R. China to Nanjing University and the Einstein Professor Program of the Chinese Academy of Sciences. He was also supported by the Canada Research Chair program, a Visiting Distinguished Professorship in the Department of Biology and Chemistry and State Key Laboratory in Marine Pollution, City University of Hong Kong.

#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.envint.2016.09.018>.

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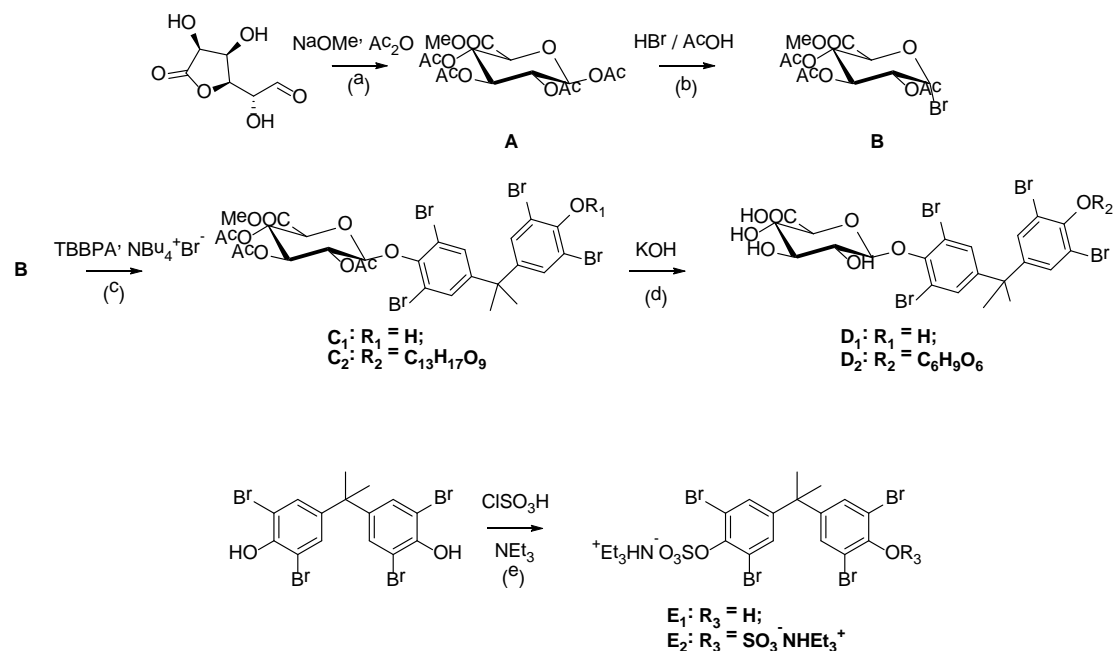


**Glucuronide and Sulfate Conjugates of Tetrabromobisphenol A (TBBPA):  
Chemical Synthesis and Correlation between Their Urinary Levels and  
Plasma TBBPA Content in Voluntary Human Donors**

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*Supplementary Information*

## Synthesis, purification and characterization of the tetrabromobisphenol-A glucuronide and sulfate conjugates



Scheme S1. General synthetic procedure of glucuronide and sulfate conjugates of tetrabromobisphenol-A: (a) NaOMe, Ac<sub>2</sub>O, perchloric acid; (b) HBr in HOAc (33%), DCM; (c) tetrabromobisphenol-A, NBu<sub>4</sub><sup>+</sup>Br<sup>-</sup>, CHCl<sub>3</sub>, K<sub>2</sub>CO<sub>3</sub>, H<sub>2</sub>O; (d) KOH, MeOH; (e) *N,N*-diethylaniline, ClSO<sub>3</sub>H, CHCl<sub>3</sub>, NEt<sub>3</sub>

### Materials.

All starting materials, tetrabromobisphenol-A, D-(+)-glucuronic acid  $\gamma$ -lactone, hydrogen bromide in glacial acetic acid (33%), acetic anhydride, potassium carbonate, tetrabutylammonium bromide, ammonium hydroxide, potassium hydroxide, sodium acetate, sodium methoxide (NaOMe), perchloric acid were purchased from commercial sources (Sigma – Aldrich, Acros Organics, or Wako) and were used as received unless stated otherwise. Solvents used for synthesis were of analytical grade while solvents used for

chromatographic analysis were of HPLC or LC-MS grade. Dichloromethane was distilled from anhydrous calcium hydride prior to use.  $^{13}\text{C}_{12}$ -Tetrabromobisphenol-A was purchased from Wellington Lab.  $^{13}\text{C}_{12}$ -Tetrachlorobisphenol-A was obtained from Cambridge Isotope Laboratories. Oasis<sup>®</sup> WAX cartridges (6 mL / 150 mg) and Sep-Pak Florisil (3 mL / 500 mg) were obtained from Waters Corp. *N,O*-Bis(trimethylsilyl)trifluoroacetamide was purchased from Supelco. LabAssay<sup>™</sup> Creatinine was obtained from WAKO. Creatinine was obtained from WAKO. Artificial urine matrix was prepared according to the literature (Wang et al., 2001).

### ***Instrumentation.***

NMR spectra were recorded by using a Bruker AV400 (400 MHz) FT-NMR spectrometer. Electrospray (ESI) mass spectra were measured by using a PE SCIEX API 365 LC/MS/MS system and Applied Biosystems SCIEX QSTAR Elite hybrid quadrupole/time-of-flight (Q-TOF) tandem high-resolution mass spectrometer. Elemental analyses were carried out on a Vario EL III CHN elemental analyzer. Purifications were done by HPLC using a Waters 515 HPLC isocratic pump and Waters 2487 dual  $\lambda$  absorbance detector (Milford, MA). Separation was performed on a Xbridge Prep C18 100 mm  $\times$  19 mm i.d.  $\times$  5  $\mu\text{m}$  preparative column at a flow rate of 5 mL/min and water : methanol (1:1 v/v) and water : acetonitrile (1:1, v/v) as the mobile phases. Quantifications of glucuronide and sulfate

conjugates of TBBPA were performed with a HPLC–ESI-MS/MS system using an Agilent 1290 Series HPLC (Agilent Technologies, Waldbronn, Germany) coupled to a MDS Sciex API 3200 QTrap triple quadrupole / linear ion trap mass spectrometer with a Turbo V ion spray source (Applied Biosystems, Foster City, CA, USA). To improve sensitivity and selectivity, analytes were detected in the Multiple Reaction Monitoring (MRM) mode with the  $d_{\text{well}}$  time of 70 ms. The ionization source parameters were as follow: ion spray voltage: -4500 kV; curtain gas ( $\text{N}_2$ ): 15 psig; collision gas ( $\text{N}_2$ ), high; temperature of ionization source, 550 °C; ion source gas 1 (nebulizer gas), 60 psig; ion source gas 2 (heater gas), 50 psig. Declustering potential (DP), entrance potential (EP), collision energy (CE) and collision cell exit potential (CXP) of all analytes were optimized to obtain maximum sensitivity.

The chromatographic separation was performed using a Waters XBridge™ C18 2.5  $\mu\text{m}$  3.0 mm  $\times$  50 mm column. A guard column XBridge™ C18 2.5  $\mu\text{m}$  3.0  $\times$  20 mm was placed in front of the analytical column. Separation was obtained using gradient elution at a flow rate of 300  $\mu\text{L}/\text{min}$ , with solvent A (Milli-Q contains 0.1%  $\text{NH}_4\text{OH}$ ) and solvent B (acetonitrile contains 0.1%  $\text{NH}_4\text{OH}$ ) at the composition of A:B (99:1, v/v) at  $t = 0$  to  $t = 5$  min, changed linearly to 15:85 (v/v) in a period of 5 min then held at such composition for a further 5 min. After the separation, the eluent composition was switched back to 99:1 (v/v) and held for 20 min before the next injection. The injection volume was 10  $\mu\text{L}$ .

Identification and quantifications of the parent tetrabromobisphenol-A,  $^{13}\text{C}_{12}$ -tetrabromobisphenol-A (recovery standard) and  $\text{d}_{10}$ -pyrene (internal standard) were performed on a gas chromatography (GC, Bruker 450-GC gas chromatograph) equipped with a tandem mass-selective detector (MS, Bruker 320-MS triple quadrupole mass spectrometer), using electron ionization (EI). The temperature of GC injector was set to 280 °C with injecting volume of 2  $\mu\text{L}$  under splitless mode. TBBPA and related analytes were analyzed by a 30 m  $\times$  0.25 mm  $\times$  0.25  $\mu\text{m}$  VF-5MS column. The temperature program was as follows: 110 °C for 1 min; 20 °C / min to 300 °C held for 10 mins. Helium was used as carrier gas and its flow rate was adjusted to 1.5 mL/min. The total running time for each analysis was 20.5 mins. Mass spectrometric parameters were set as following: the ion source temperature was set at 200 °C whereas temperature of manifold was set at 40 °C. Electron impact voltage was set to 70 eV. Argon at 1.5 mTorr was used as the collision gas in the collision cell. Solvent delay was set to 4.0 min. The detector was an electron multiplier with extended dynamic range (EDR). Mass transitions for each of the targeted compounds are shown in Table S1. Quantification of each analyte was based on the peak area using one target and one quantifier ion.

### ***Intermediates A & B.***

Intermediates A and B were prepared according to published procedures (Bollenback et al., 1955; Pilgri et al., 2010)

### ***Tetrabromobisphenol-A mono-2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranosiduronic acid methyl ester (C<sub>1</sub>)***

A solution of 1-bromo-2,3,4-tri-O-acetyl- $\alpha$ -D-glucopyranuronic acid, methyl ester (1.0 g, 2.52 mmol) in chloroform (20 mL) was vigorously stirred, heated under reflux at 70 °C overnight with a solution of TBBPA (4.11 g, 7.55 mmol), tetrabutylammonium bromide (0.41 g, 1.26 mmol) and potassium carbonate (2.19 g, 15.8 mmol) in aqueous solution (30 mL). The reaction mixture was stirred and heated under reflux. The reaction mixture was cooled and water was added. Two layers were separated, and the organic layer was isolated and washed with saturated K<sub>2</sub>CO<sub>3</sub> solution twice, followed by distilled water, dried over anhydrous magnesium sulfate, and finally evaporated in *vacuo*. The yellow syrup was purified by flash silica column chromatography by a solvent system of ethyl acetate : petroleum ether (2:1) to afford tetrabromobisphenol-A mono-2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranosiduronic acid methyl ester as white solid (1.38 g, 63.8 %).

***Tetrabromobisphenol-A di-2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranosiduronic acid methyl ester (C<sub>2</sub>)***

The synthetic method was similar to that of tetrabromobisphenol-A mono-2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranosiduronic acid methyl ester (C<sub>1</sub>) except that the mole ratio of TBBPA to 1-bromo-2,3,4-tri-O-acetyl- $\alpha$ -D-glucopyranuronic acid methyl ester used was 1 : 3. The reaction mixture was heated under reflux at 70 °C overnight. The yellow syrup was purified by flash silica column chromatography using a solvent system of ethyl acetate:petroleum ether (1:1) to afford tetrabromobisphenol-A di-2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranosiduronic acid methyl ester as a white solid (4.35 g, 47.9 %).

***Tetrabromobisphenol-A mono-glucuronide (D<sub>1</sub>)***

Tetrabromobisphenol-A mono-2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranosiduronic acid methyl ester (0.70 g, 0.82 mmol) was dissolved in MeOH (20 mL). Potassium hydroxide (0.48 g, 8.20 mmol) was added and the mixture was stirred overnight. The precipitate was collected and re-dissolved in a mixture of formic acid and methanol (1:9). The solution was then collected and evaporated in *vacuo* and the white solid was collected as the crude product of TBBPA mono-glucuronide (0.42 g). This crude product was subjected to HPLC purification. ESI-MS (-ve): 718.9 (M-H<sup>-</sup>), 543.7 (M-H-C<sub>6</sub>O<sub>6</sub>H<sub>8</sub><sup>-</sup>). <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O):  $\delta$  Q 7.542 (s, 2H), 7.412 (s, 2H), 5.304 – 5.285 (d, 1H, *J* = 7.6Hz), 3.742 – 3.761 (m, 1H), 3.670 – 3.596

(m, 3H), 1.592 (s, 6H). HRMS (-ve) calcd for  $C_{21}H_{20}Br_4O_8$ : 718.77744; found 718.7770.

Elemental analysis calcd(%) $C_{21}H_{20}Br_4O_8$ : C 35.03, H 2.80, N 0.00; found C 35.21, H 2.84 N 0.01.

### ***Tetrabromobisphenol-A di-glucuronide (D<sub>2</sub>)***

The synthetic method was similar to that of tetrabromobisphenol-A mono-glucuronide (D<sub>1</sub>) except that tetrabromobisphenol-A di-2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranosiduronic acid methyl ester (C<sub>2</sub>) was used instead of tetrabromobisphenol-A mono-2,3,4-tri-O-acetyl- $\beta$ -D-glucopyranosiduronic acid methyl ester (C<sub>1</sub>). Also, the amount of potassium hydroxide was 2 times larger than that in the synthesis of (D<sub>1</sub>). The solution was then collected and evaporated in *vacuo* and the white solid was collected as the crude product of TBBPA mono-glucuronide (0.63 g). ESI-MS (-ve): 895.0 (M-H<sup>-</sup>), 719.0 (M-H-C<sub>6</sub>O<sub>6</sub>H<sub>8</sub><sup>-</sup>), 542.9 (M-H-C<sub>12</sub>O<sub>12</sub>H<sub>16</sub><sup>-</sup>). <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O):  $\delta$  7.538 (s, 4H), 5.309 – 5.289 (d, 2H, *J* = 8.0Hz), 3.787 – 3.744 (m, 2H), 3.664 – 3.597 (m, 6H), 1.618 (s, 6H). HRMS (-ve) calcd for  $C_{27}H_{28}Br_4O_{14}$ : 894.80965; found 894.8117. Elemental analysis calcd(%) $C_{27}H_{28}Br_4O_{14}$ : C 36.19, H 3.15, N 0.00; found C 36.28, H 3.11 N 0.02.

### ***Tetrabromobisphenol-A mono-sulfate (E<sub>1</sub>)***

Synthesis of sulfate ester was in accordance with the literature with modifications.<sup>52</sup>



Diethylaniline (3.65 mL, 22.9 mmol) in chloroform (4 mL) was stirred at -40 °C. Chlorosulphonic acid (0.66 mL, 9.9 mmol) was added over a period of 1 hour and the temperature of the mixture was maintained below 10 °C. Tertabromobisphenol-A (5 g, 9.2 mmol) in chloroform (20 mL) was added all at once. The resultant mixture was stirred overnight at room temperature. Triethylamine was then added to the reaction mixture until basic pH. The white precipitate was collected by filtration as the crude product of TBBPA mono-sulfate mono-triethylamine salt, and chlorosulphonic acid triethylamine salt (8.2 g). This crude product was subjected to further purification by HPLC. ESI-MS (-ve): 623.0 (M-H-NEt<sub>3</sub><sup>-</sup>), 543.0 (M-H-SO<sub>3</sub>-NEt<sub>3</sub><sup>-</sup>). <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ Q 7.577 (s, 2H), 7.404 (s, 2H), 3.236 – 3.182 (q, 6H, *J* = 7.2Hz), 1.597 (s, 6H), 1.268 – 1.304 (t, 9H, *J* = 7.2Hz). HRMS (-ve) calcd for C<sub>15</sub>H<sub>11</sub>Br<sub>4</sub>O<sub>5</sub>S: 622.70201; found 622.6995. Elemental analysis calcd(%)C<sub>21</sub>H<sub>26</sub>Br<sub>4</sub>NO<sub>5</sub>S: C 34.78, H 3.75, N 1.93; found C 34.86, H 3.71 N 1.86.

### ***Tetrabromobisphenol-A di-sulfate (E<sub>2</sub>)***

The synthetic method was similar to that of tetrabromobisphenol-A mono-sulfate (E<sub>1</sub>) except that the amount of chlorosulphonic acid used was doubled compared to the synthesis of (E<sub>1</sub>). The crude product obtained (3.62 g) was subjected to further purification by HPLC. ESI-MS (-ve): 803.9 (M-H-NEt<sub>3</sub><sup>-</sup>), 623.5 (M-H-SO<sub>3</sub>-NEt<sub>3</sub><sup>-</sup>), 543.5 (M-H-S<sub>2</sub>O<sub>6</sub>-N<sub>2</sub>Et<sub>6</sub><sup>-</sup>). <sup>1</sup>H NMR (400 MHz, MeOD-D<sub>4</sub>): δ Q 7.443 (s, 24H), 3.210 – 3.155 (q, 12H, *J* = 7.6Hz), 1.641 (s,

6H), 1.301 – 1.264 (t, 18H,  $J = 7.6\text{Hz}$ ). HRMS (-ve) calcd for  $\text{C}_{21}\text{H}_{26}\text{Br}_4\text{NO}_8\text{S}_2$ : 803.77931; found 803.7812. Elemental analysis calcd(%) $\text{C}_{27}\text{H}_{42}\text{Br}_4\text{N}_2\text{O}_8\text{S}_2$ : C 35.78, H 4.67, N 3.09; found C 35.87, H 4.72 N 3.00.

### ***Purification of glucuronide and sulfate conjugates of TBBPA.***

Crude mono- and di-glucuronide and mono- and di-sulfate of TBBPA conjugates were purified by HPLC using a Waters 515 HPLC isocratic pump and Waters 2487 dual  $\lambda$  absorbance detector (Milford, MA). Separation was performed on a Xbridge Prep C18 100 mm  $\times$  19 mm i.d.  $\times$  5  $\mu\text{m}$  preparative column at a flow rate of 5 mL/min and water : methanol (1:1 v/v) and water : acetonitrile (1:1, v/v) as the mobile phases. This HPLC separation procedure was repeated until sufficient amount of each of the conjugates were obtained.

### ***Quality Control and Quality Assurance.***

Procedural blanks and matrix spikes were analyzed for every batch of 18 samples in analysis of blood TBBPA and urinary TBBPA and TBBPA phase II conjugates.

Surrogate standards were used to quantify the concentration of TBBPA using mean relative response factors determined from standard calibration during analysis of human plasma samples.  $^{13}\text{C}_{12}$ -TBBPA was used as surrogate standards. All equipment was rinsed

with acetone and hexane to avoid sample contamination. One laboratory blank and one matrix spike were analyzed for each batch of 18 samples to check for interferences or contamination from solvent and glassware. No TBBPA was detected in blank analysis. Estimation of the method detection limit (MDL) has to be carried out using a lower spike concentration and it is determined by a consecutive analysis of the series of  $n$  spiked samples according to the following equation:

$$\text{MDL} = t \times \sigma$$

where  $\sigma$  is the standard deviation of the data and  $t$  is the compensation factor from the Student's  $t$ -Table with  $n - 1$  degrees of freedom at a confidence interval of 95%. MDLs of TBBPA and  $^{13}\text{C}_{12}$ -TBBPA in human plasma sample were found to be 0.005 ng/g l.w. Recoveries of TBBPA and its surrogate standards  $^{13}\text{C}_{12}$ -TBBPA in human plasma were within 81 – 107%. Those of the spiked matrices were 74 – 119%.

### ***Statistical Analysis.***

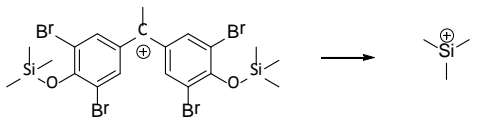
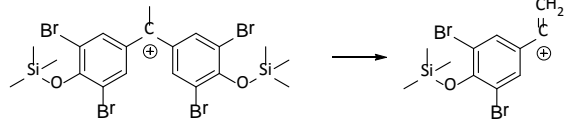
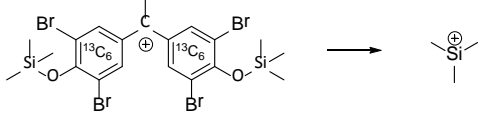
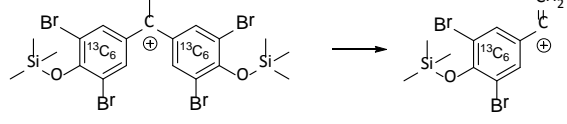
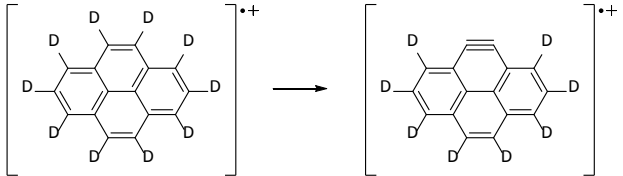
All statistical analyses were carried out with the SPSS 16 (SPSS Inc., Chicago, IL), Prism 2.01 (GraphPad Software, Inc.) and Sigmapstat 3.5 (Sigmapstat, Jandel Scientific, CA). Normality of the distributions was assessed by the Kolmogorov-Smirnov test. Logarithm or natural-logarithm transformation was used to obtain normally distributed data sets for parametric statistical tests. Logarithm, natural-logarithm, arcsine, square root, square root or

cubic root transformation was used to obtain normally distributed data sets for parametric statistical tests. Student's *t*-test was used to statistically analyze the concentration of TBBPA as well as TBBPA phase II conjugates in both human plasma and urine between male and female if the data passed for normality and equal variance. If the data were not normally distributed, nonparametric Mann-Whitney Rank Sum test was used for comparison between male and female. One-way ANOVA (parametric) or ANOVA on ranks (non-parametric) was used to comparison of the analytes among different age categories if applicable. As most variables showed a skewed distribution, natural-log transformation on urinary TBBPA conjugates was taken to reduce their skewness for all statistically analysis. Pearson product moment was used to examine relationships between  $\ln[\text{TBBPA}]$  in human plasma and  $\ln\Sigma[\text{TBBPA phase II metabolites}] / \ln[\text{TBBPA}]$  in human urine. A  $P < 0.05$  was considered statistically significant.

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Table S1. Proposed fragmentation assignments of mass spectra of compound (TBBPA) analyzed by GC-MS/MS

Compound	Transition ( $m/z$ ), Collision energy (V) & Assignments	
Tetrabromobisphenol-A	<p><math>673 \rightarrow 73</math>, 34V [M - C<sub>15</sub>H<sub>14</sub>O<sub>2</sub> - Si - C<sub>3</sub>H<sub>9</sub>]<sup>+</sup> <math>m/z = 673</math></p> 	<p><math>673 \rightarrow 365</math> 10V [M - C<sub>15</sub>H<sub>14</sub>O<sub>2</sub> - Si - C<sub>3</sub>H<sub>9</sub>]<sup>+</sup> <math>m/z = 673</math></p> 
<sup>13</sup> C <sub>12</sub> - Tetrabromobisphenol-A	<p><math>685 \rightarrow 73</math> 34V [M - C<sub>15</sub>D<sub>14</sub>O<sub>2</sub> - Si - C<sub>3</sub>H<sub>9</sub>]<sup>+</sup> <math>m/z = 685</math></p> 	<p><math>685 \rightarrow 371</math> 10V [M - C<sub>15</sub>H<sub>14</sub>O<sub>2</sub> - Si - C<sub>3</sub>H<sub>9</sub>]<sup>+</sup> <math>m/z = 685</math></p> 
D <sub>10</sub> -pyrene (internal standard)	<p><math>212 \rightarrow 208</math> 36V [M - D<sub>2</sub>]<sup>++</sup> <math>m/z = 212</math></p>  <p><math>m/z = 208</math></p> 