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Profiles, variability, and predictors of urinary benzotriazoles and benzothiazoles in pregnant women from Wuhan, China

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ABSTRACT

Background: Benzotriazoles (BTRs) and benzothiazoles (BTHs) are emerging contaminants with high production volume worldwide, which exhibit potential health risk to human. To date, little is known about the exposure of BTRs and BTHs (BTs) on human, especially in the context of pregnancy.
Objectives: We aimed to characterize the exposure profiles, temporal variability, and potential predictors of urinary BTs during pregnancy.
Methods: Between 2014 and 2015, we recruited 856 pregnant women in Wuhan who provided urine samples at three trimesters (13.1 ± 1.1, 23.7 ± 3.2, and 35.7 ± 3.4 gestational weeks). We measured the urinary concentrations of five BTRs (1-H-benzotriazole, 1-hydroxy-benzotriazole, xylyltriazole, tolyltriazole, 5-chloro-1-H-benzotriazole) and five BTHs (benzothiazole, 2-hydroxy-benzothiazole, 2-methylthio-benzothiazole, 2-amino-benzothiazole, 2-thiocyanomethylthio-benzothiazole) to characterize the exposure profiles of BTs. We calculated the intra-class correlation coefficients (ICCs) to assess the temporal variability and investigated potential predictors of urinary BTs by using the mixed models.
Results: Most of the targeted BTs were detected in over 50% of urine samples, except for 5-chloro-1-H-benzotriazole (9.3%) and 2-thiocyanomethylthio-benzothiazole (1.4%). The predominant BTRs in urine was 1-hydroxy-benzotriazole [Geometric Mean (GM): 0.77 ng/mL]. Benzothiazole was the major derivative in urine samples with a GM concentration of 1.6 ng/mL. Correlations among BTHs (r = 0.04–0.39) were higher than that among BTRs (r = 0.02–0.14). The exposure pattern was constant at low level and co-exposure to all the targeted compounds was infrequent during pregnancy. Urinary concentrations of BTRs exhibited considerable within-subject variation (ICCs: 0.12–0.56) during pregnancy. Relatively high temporal reliability was observed for urinary concentrations of BTHs with ICCs ranging from 0.42 to 0.85. It was found that parity, household income, pregnancy occupational status, sampling season and menstrual cycle were associated with urinary concentrations of BTs in pregnant women ($P < 0.05$).
Conclusions: To the best of our knowledge, this is the first study to report the exposure profiles, variability and predictors of urinary BTs among pregnant women. Exposure assessment using multiple samples is essential in reducing measurement errors and identifying susceptible window of exposure in etiological studies. The

Abbreviations: BTRs, benzotriazoles (substances that contain 1,2,3-benzotraizole skeleton); BTHs, benzothiazoles (substances that contain 1,3-benzothiazole skeleton); BTs, BTRs and BTHs; 1-H-BTR, 1-H-benzotriazole; 1-OH-BTR, 1-hydroxy-benzotriazole; XTR, xylyltriazole or 5,6-dimethyl-1-H-benzotriazole; TTR, tolyltriazole (a mixture of isomers of 4-methyl-1-H-benzotriazole and 5-methyl-1-H-benzotriazole); 5-Cl-H-BTR, 5-chloro-1-H-benzotriazole; BTH, benzothiazole; 2-OH-BTH, 2-hydroxy-benzothiazole; 2-MeS-BTH, 2-methylthio-benzothiazole; 2-NH_2-BTH, 2-amino-benzothiazole; 2-SCNMeS-BTH, 2-thiocyanomethylthio-benzothiazole; ICC, intra-class correlation coefficient

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potential predictors of urinary BTs raised concerns on tracing exposure routes and eliminating confounding variables in future studies.

1. Introduction

Benzotriazoles (BTRs) that contain 1,2,3-benzotriazole skeleton and benzothiazoles (BTHs) composed of 1,3-benzothiazole skeleton are emerging contaminants with high production volume. BTRs and BTHs (BTs) are widely used in a variety of industrial and household items, such as flaming and corrosion inhibitors, fungicides, dishwasher detergents, antifogging fluids and vulcanization accelerators (Pillard et al., 2001; Kloepfer et al., 2005). The commercially manufactured BTs may ultimately be released into the environment, with the detectable concentrations of BTs in indoor dust, indoor air, tap water, sewage, seafood and textiles (Wang et al., 2016; Reemtsma et al., 2010; Wang et al., 2013; Wan et al., 2016; Avagyan et al., 2013; Karthikraj and Kannan, 2017; Liao et al., 2018). The ubiquity of the contaminants provided potential routes of human exposure to BTs, including inhalation, ingestion and dermal absorption through textiles (Wan et al., 2016; Wang et al., 2013; Avagyan et al., 2015; Liu et al., 2017; Asheim et al., 2019). However, only a limited number of studies have assessed the exposure profiles of BTs among humans (Asimakopoulos et al., 2013b; Li et al., 2018; Wang et al., 2015).

Previous biomonitoring studies have demonstrated the occurrence of BTs in a variety of human tissues and body fluids (Ferrario et al., 1985; Li et al., 2018; Lee et al., 2015; Asimakopoulos et al., 2013b; Wang et al., 2015). The occurrence of BTs in amniotic fluid and breast milk highlights the risk for direct fetal exposure (Li et al., 2018; Lee et al., 2015). Developmental toxicity of BTs was reported in marine animals, when the zebrafish embryos were treated with 4-methyl-1-H-benzotriazole at 50 μg/mL, cardiac function abnormalities such as pericardial edema and poor blood circulation were observed (Damalas et al., 2018). This evidence indicated that gestational exposure to BTs may pose adverse health effects on fetus development (Liao et al., 2018). Therefore, gestational exposure assessments are warranted to serve as the basis of studies on health effects of BTs.

Due to the high polarity and hydrophilicity, BTs were assumed to mainly excrete through urine as free and conjugated forms (Asimakopoulos et al., 2013a). A single spot time urine sample was commonly used to evaluate BTs body burden in human biomonitoring studies (Asimakopoulos et al., 2016; Asimakopoulos et al., 2013b; Li et al., 2018). The half-life of BTH was reported to be 4 days after daily i.p. administration of 30 mg/kg in genuine pigs (Wilson et al., 2008). For chemicals with such short half-life, one spot urine sample may not be sufficient to accurately capture the exposure profiles during a period of time (Perrier et al., 2016). The misclassification of exposure level in etiological studies is expected to bias the health effects (Hauser et al., 2004). Therefore, administering multiple urine samples for measurement of chemical exposure throughout the exposure window is recommended in epidemiology studies to minimize measurement bias and to explore the susceptible window of exposure (Johns et al., 2015). However, it is not always feasible to collect multiple urine samples considering the costs and the availability of participants (Galea and Tracy, 2007). Consequently, a better understanding of the temporal variability of urinary BTs could help reconcile sampling scheme with budget and the sensitivity of one spot urine sample. However, little is known about the ability of a single spot urine sample to represent an individual's exposure level of BTs over a period of time.

In order to trace exposure routes and eliminate confounding variables of health effects studies, it is essential to identify factors that may be associated with urinary concentrations of BTs. However, constrained by the small sample size, the implications of demographic factors on BTs exposure were not well understood in previous studies, especially in the context of pregnancy (Asimakopoulos et al., 2013b; Li et al., 2018). In the present study, urinary concentrations of BTs were measured in 2568 samples collected from 856 pregnant women at three different trimesters to characterize the exposure profiles of BTs during pregnancy. We further examined the temporal variability and potential predictors of urinary BTs during pregnancy. The results may help inform researchers to trace exposure routes and provide insights into optimizing sampling schemes and eliminating confounding variables in etiology studies.

2. Materials and methods

2.1. Study population

This study was based on data collected from an ongoing prospective birth cohort in a major hospital in Wuhan, China: Wuhan Women and Children's Medical Care Center. Women that met the following criteria were recruited in the birth cohort at their early pregnancy: 1) city residents that would not move out of the city in the foreseeable future; 2) conceived a singleton baby; 3) < 16 weeks of gestation; 4) would receive all gestational care and delivery at the study hospital; 5) volunteered to take part in the study and provide urinary samples during pregnancy. Between 2014 and 2015, we recruited 1244 participants. In the present study, we focused on 856 pregnant women who provided urine samples at the first, second and third trimesters. We did not observe significant demographic differences between the participants (n = 856, 69%) and those who only provided one sample (n = 183, 15%) or two samples (n = 205, 16%) (Table S1), indicating that the participants in the present study may be representative of the population in the prospective birth cohort. The research protocol was approved by the ethics committees of the Tongji Medical College, Huazhong University of Science and Technology and the study hospital. All participants received a detailed explanation of the study and provided a written informed consent at enrollment.

2.2. Data and sample collection

Demographic characteristics (e.g., maternal age, pregnancy occupational status, menstrual cycle, education and household income) were obtained via a face-to-face questionnaire conducted by well-trained nurses. Irregular menstrual cycle was defined as variations in length for > 7 days between cycles (Fraser et al., 2011). Information regarding last menstrual period, parity and gravity were retrieved from the maternity clinic medical records.

Urine samples collected at up to three trimesters from each individual were used to evaluate the exposure level and estimate the variation during pregnancy. Gestational weeks were estimated based on last menstrual period and further confirmed by their first-trimester ultrasound examination. The mean gestational weeks of the three trimesters for sampling were 13.1 ± 1.1, 23.7 ± 3.2, and 35.7 ± 3.4 weeks, respectively. Urine samples were collected and stored in polyethylene containers at −20 °C until further analysis.

2.3. Chemicals and reagents

Urine samples were analyzed for five BTRs [1-H-benzotriazole (1-H-BTR), 1-hydroxy-benzotriazole (1-OH-BTR), xylyltriazole (XTR), tolyltriazole (TTR, a mixture of isomers of 4-methyl-1-H-benzotriazole and 5-methyl-1-H-benzotriazole), 5-chloro-1-H-benzotriazole (5-Cl-1-H-BTR)] as well as five BTHs [benzothiazole (BTH), 2-hydroxy-benzothiazole (2-OH-BTH), 2-methylthio-benzothiazole (2-MeS-BTH), 2-amino-benzothiazole (2-NH_2-BTH), and 2-thiocyanomethylthio-

benzothiazole (2-SCNMeS-BTH)]. Detailed information of analytical standards and reagents have been reported previously (Li et al., 2017). Stock solution of internal standard (1-H-BTR-*d4*, 400 ng/mL) and mixed standard (1000 ng/mL) was fortified in methanol (BDH PROLABO, Fontenay-sous-Bois, France). The stock solution was stored at −20 °C prior to the usage.

2.4. Sample pretreatment

The total amount of BTs (free plus conjugated) was determined following the procedures reported in previous studies with minor modification (Asimakopoulos et al., 2013a; Li et al., 2017). In summary, 1 mL of urine was introduced into a polypropylene conical centrifuge tube and spiked with *β*-glucuronidase/sulfatase (10 μL, 200 units/mL). The enzymatic de-conjugation resulted in hydrolysis to release the free derivatives. The samples were subsequently buffered with 200 μL of ammonium acetate (7.7 g of NH_4-HAc dissolved in 100 mL of deionized water with 6 mL of HAc adjusting the PH to 4.5–5.0) and incubated in a shaking water bath at 37 °C overnight. Isotopically labeled internal standard (1-H-BTR-*d4*, 0.2 ng each) was spiked into the samples before extraction. A volume of 3 mL solvents (methyl tert-butyl ether: ethyl acetate, 5:1) was added into the matrix to extract the analytes. Then the samples were subjected for ultrasound treatments and shaken for 30 min. After ultrahigh speed centrifugation, the supernatant organic phase was transferred into a glass tube. The liquid-liquid extraction procedure was repeated twice and the combined extracts were evaporated to dryness under a gentle stream of high-purity nitrogen. The residue was subsequently reconstituted with 200 μL of solvent (ACN: H_2O, 6:4). After being gently vortex-mixed for 0.5 min, the mixture was sonicated for 10 min. To precipitate proteins, an ultracentrifugation step was taken at 15000 rpm for 10 min at 8 °C. The supernatant (180 μL) was introduced into the glass vial insert for instrumental analysis.

2.5. Instrumental analysis

Total BTs were quantified by ultra-performance liquid chromatography coupled with triple quadrupole mass spectrometry (UPLC-MS/MS) in positive electrospray ionization (ESI^+) mode (Waters, TQXS). Chromatography separation was achieved using Acquity BEH C18 column (100 mm * 2.1 mm, 1.7um, Thermo, MA, USA) with mobile phase: 0.01% formic acid in water (A) and ACN (B) at a flow rate of 0.25 mL/min. The column temperature was set as 40 °C. The elution gradient was 0.0–0.5 min, 10% B; 9.5 min, 83% B; 10–12 min, 100% B; 12.2–15 min, 10% B. The chromatography for separation of the ten types of BTs in a calibration standard solution (50 ng/mL) was shown in Fig. S1. Multiple reaction monitor (MRM) transitions and the optimized MS/MS parameters for the analysis of BTs were listed in Table S2.

2.6. Quality control and quality assurance

For MRM detection, the two most intense transitions of each compound were recorded. The compounds were identified through comparing the ratio of the precursor ion to produced ion transitions with those of the standards. The tolerance limit of the variation between the ion ratios in urine samples and that in the standards were below 20%. The retention time (RT) and relative retention time (RRT) to internal standard were monitored and compared with those found for the standards to avoid false positive detection. For determination of urinary BTs, the total run time was 15 min and the variation of RT and RRT were below 2%. The specific fragment ion with the highest abundance in the spectrum was selected as the quantification ion. Quantification was applied with internal standard method.

Contamination that may arise from laboratory materials and solvents was evaluated by procedure blank detection (1 mL of deionized water). Extracted pooled urine samples ($n = 50$) were injected to monitor potential instrumental response shifting. We ran matrix spiked samples (pooled urine samples spiked with the mixture of standards) with each batch of samples to ensure analytical precision and accuracy.

The limits of detection (LODs) were defined as the concentration with a signal-to-noise ratio of 3 if the specific BTs were not detected in procedure blanks ($n = 3$). For the BTs detected in the blanks, LODs were calculated as the mean concentration plus three times the standard deviation of the blanks. All urine samples with extremely high concentrations of BTs were reanalyzed to confirm the values. Table S3 listed the linear range, correlation coefficient, LODs, limits of quantitation (LOQs), accuracy and recovery of the method. All *r*-squared values were > 0.999 (except for BTH, $r^2 = 0.995$), which ensured good predictability. Recoveries of targeted analytes ranged from 62% ± 9.4% to 140% ± 15%, and the inter-day variation was below 10%.

Specific gravity (SG) was measured by a hand-held digital refractometer (Atago Co. Ltd., Tokyo, Japan) at room temperature to adjust for the urinary dilution. The SG-adjusted concentrations were calculated using the formula $Pc = P\ [(1.011 - 1) / (SG - 1)]$, where Pc is the SG-adjusted concentration (ng/mL), P is the observed concentration, 1.011 is the median SG in our population and SG is the specific gravity of the urine sample (Hauser et al., 2004).

2.7. Statistical analysis

Geometric mean (GM) and selected percentiles were calculated to describe the distribution profiles of urinary BTs. Normality assumption was checked with a Shapiro-Wilk test and a quantile-quantile plot. Spearman correlation coefficients were used to estimate pairwise associations among SG-adjusted concentrations of urinary BTs. We further performed the percentile analysis to characterize the co-exposure pattern of BTs at each trimester as well as the exposure to specific BTs across the period of pregnancy (Gao et al., 2017). Intraclass correlation coefficient (ICC), which was defined as the ratio of between-subject variance to total variance, was computed to evaluate the temporal variability of the repeated measures over time. Only those samples with measurable concentrations were studied in the ICC analysis using the two-way mixed effect ANOVA model.

The linear mixed model was used to examine the associations of urinary BTs with demographic factors, such as maternal age, pregnancy occupational status, household income and menstrual cycle, etc. Concentrations below the LODs were substituted with a value equal to the LOD divided by the square root of two (Hornung and Reed, 1990). Natural logarithm transformed concentrations of urinary BTs were used as the dependent variable, with a separate model for each independent variable. Selected factors were modeled as fixed effects, whereas specific gravity was modeled as a time varying factor. Random effects for subjects were introduced in the models to account for correlations among repeated samples collected on the same individual over time. Statistical analyses were performed using IBM SPSS Statistics, version 20 (Chicago, IL) with the statistical significance set at two-way $P < 0.05$.

3. Results

3.1. Population characteristics

Demographic characteristics of the participants are shown in Table 1. The mean age of the pregnant women was 28.6 ± 3.3 years at enrollment, ranging from 20 to 44 years. Most of the study population were primiparous (86%). The majority of the subjects reported to have high school education or above (94%). Approximately 43% of the women had household income above 100,000 Chinese Yuan per year and 85% of women were employed during pregnancy. The number of samples collected in spring (28%), summer (24%), fall (22%) and winter (26%) were almost equally distributed. We also observed that

Table 1
Demographic characteristics of the participants (n = 856) from Wuhan, China (2014–2015).

Characteristics	Frequency or mean ± SD	Percentage (%)
Age (years)	28.6 ± 3.3	
< 28	475	55
≥28	381	45
Parity		
1	734	86
≥2	122	14
Education		
College (> 12 years)	671	78
High school (9–12 years)	134	16
Under high school (< 9 years)	51	6.0
Household income (Yuan/year)		
Above 100,000	364	43
50,000–100,000	323	38
Below 50,000	166	19
Missing	3	
Pregnancy occupational status		
Unemployment	93	11
Employment	732	85
Missing	31	4.0
Sampling season		
Winter (December–February)	670	26
Fall (September–November)	563	22
Spring (March–May)	717	28
Summer (June–August)	614	24
Missing	4	
Menstrual cycle		
Irregular	54	6.0
Regular	792	93
Missing	10	1.0

6.0% of subjects suffered from irregular menstrual cycle (variation ≥7 d between cycles) before pregnancy.

3.2. Profiles of urinary BTs among pregnant women

Urinary concentrations of BTs were measured in 2568 samples collected from 856 participants at three trimesters. The sum concentrations of the five BTRs and the five BTHs were denoted as ΣBTRs and ΣBTHs, respectively. Distribution profiles of the urinary BTs were displayed in Table 2. Eight of the ten BTs were detected in over 50% of the urine samples, except for 5-Cl-H-BTR (9.3%) and 2-SCNMeS-BTH (1.4%). Owing to the low detection frequency, the results of these two compounds were not informative. Therefore, further analysis of these two compounds was not performed. The most frequently detected compound was 2-MeS-BTH (92%), followed by BTH (88%). BTH was the major derivative with a geometric mean (GM) concentration of 1.6 ng/mL. The ΣBTRs in urine samples ranged from undetected to 212 ng/mL, with 1-OH-BTR (GM: 0.77 ng/mL) being the dominance, followed by 1-H-BTR (0.32 ng/mL), XTR (0.12 ng/mL) and TTR (0.11 ng/mL). Comparisons of the concentration distributions between different trimesters (13.1 ± 1.1, 23.7 ± 3.2, and 35.7 ± 3.4 weeks) were box plotted in Fig. S2. There were no statistically significant differences for the exposure profiles between the three trimesters for any of the compounds.

3.3. Correlations and exposure patterns of urinary BTs during pregnancy

The heatmap (Fig. 1) displays the pairwise correlations between SG-adjusted concentrations of urinary BTs among pregnant women. We observed that within-class correlations were higher among BTHs (r = 0.04–0.39) than that among BTRs (r = 0.02–0.14). When focusing on chemicals from different classes, there were moderate positive correlations between 1-H-BTR and 2-NH_2-BTH (r = 0.38, P < 0.01), and

Table 2
Distribution profiles of urinary BTs concentrations (ng/mL) (n = 2568) among 856 study participants.

Analytes	DF %	Percentile				Max	Mean (95% CI)	GM (95% CI)
		25th	50th	75th	95th			
1-H-BTR								
Unadjusted	60	< LOD	0.091	0.39	1.3	42	0.57 (0.51, 0.64)	0.32 (0.30, 0.34)
SG-adjusted		< LOD	0.10	0.44	1.6	36	0.74 (0.66, 0.83)	0.38 (0.35, 0.40)
1-OH-BTR								
Unadjusted	64	< LOD	0.29	0.96	3.1	210	1.8 (1.5, 2.3)	0.77 (0.73, 0.81)
SG-adjusted		< LOD	0.32	1.0	3.7	330	2.1 (1.6, 2.7)	0.86 (0.82, 0.91)
XTR								
Unadjusted	66	< LOD	0.040	0.14	0.72	22	0.30 (0.26, 0.34)	0.12 (0.11, 0.13)
SG-adjusted		< LOD	0.040	0.17	0.88	18	0.35 (0.30, 0.39)	0.13 (0.12, 0.14)
TTR								
Unadjusted	60	< LOD	0.040	0.10	0.40	7.1	0.19 (0.17, 0.20)	0.11 (0.10, 0.11)
SG-adjusted		< LOD	0.036	0.13	0.66	6.1	0.25 (0.23, 0.27)	0.13 (0.12, 0.13)
BTH								
Unadjusted	88	0.36	1.4	2.9	6.8	44	2.6 (2.4, 2.7)	1.6 (1.5, 1.7)
SG-adjusted		0.39	1.5	3.2	9.3	53	3.1 (3.0, 3.3)	1.7 (1.6, 1.8)
2-OH-BTH								
Unadjusted	81	0.037	0.27	0.82	3.4	220	1.1 (0.94, 1.4)	0.46 (0.43, 0.49)
SG-adjusted		0.029	0.28	1.0	6.6	160	2.0 (1.7, 2.3)	0.54 (0.50, 0.58)
2-MeS-BTH								
Unadjusted	92	0.12	0.30	0.58	1.4	5.3	0.52 (0.50, 0.54)	0.33 (0.32, 0.35)
SG-adjusted		0.10	0.30	0.66	2.3	15	0.71 (0.66, 0.75)	0.37 (0.35, 0.39)
2-NH_2-BTH								
Unadjusted	57	< LOD	0.018	0.053	0.16	1.5	0.080 (0.074, 0.086)	0.052 (0.050, 0.054)
SG-adjusted		< LOD	0.017	0.063	0.21	8.3	0.10 (0.092, 0.12)	0.060 (0.057, 0.063)
2-SCNMeS-BTH								
Unadjusted	1.4	< LOD	< LOD	< LOD	< LOD	0.16	0.037 (0.035, 0.038)	0.032 (0.031, 0.034)
SG-adjusted		< LOD	< LOD	< LOD	< LOD	0.66	0.048 (0.045, 0.052)	0.035 (0.033, 0.037)
5-Cl-H-BTR								
Unadjusted	9.3	< LOD	< LOD	< LOD	0.020	0.24	0.030 (0.026, 0.034)	0.026 (0.024, 0.028)
SG-adjusted		< LOD	< LOD	< LOD	0.028	0.53	0.052 (0.042, 0.066)	0.037 (0.032, 0.043)

Abbreviations: DF, detection frequency; Max, maximum; GM, geometric mean; CI, confidence interval; SG, specific gravity; LOD, limit of detection.

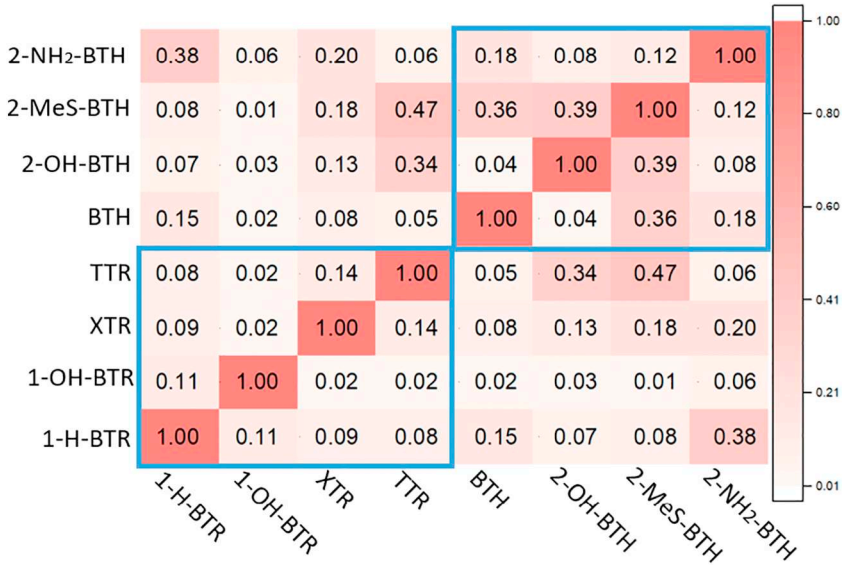


Fig. 1. Heatmap of Spearman correlations between SG-adjusted concentrations of urinary BTs.

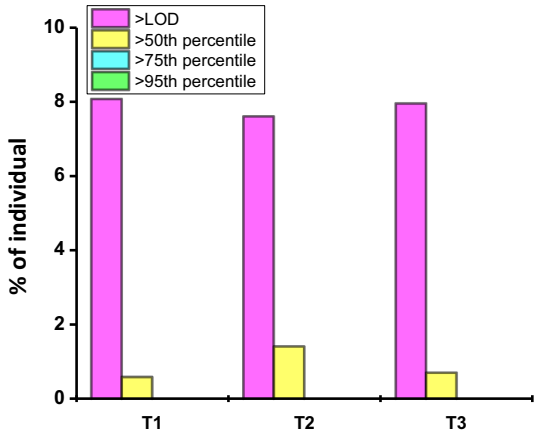
between TTR and 2-OH-BTH ($r = 0.34$, $P < 0.01$), 2-MeS-BTH ($r = 0.47$, $P < 0.01$). Along these lines, we further performed the percentile analysis to characterize the co-exposure pattern of BTs at each trimester (Fig. 2A). The proportion of participants had detectable concentrations for all the 8 compounds at the first, second, and third trimester were 8.1% ($n = 69$), 7.6% (65) and 7.9% (68), respectively. While only a small percentage of participants had the exposure levels above the 50th percentile for all the analyzed compounds (0.58%, 1.4% and 0.70% for the first, second and third trimester, respectively). When focusing on each compound across pregnancy (Fig. 2B), the exposure pattern was likely to be constant at low level during pregnancy. For example, 66% of participants had detectable concentration of urinary BTH over the three trimesters. The proportion of participants had urinary concentration of BTH above the 50th percentile, 75th percentile and 95th percentile during pregnancy were 28%, 9.5% and 0.82%, respectively.

3.4. Variability of urinary BTs during pregnancy

The ICCs were represented on volume- and SG-adjusted bases, respectively (Table 3). ICCs approaching zero indicate low reproducibility during the study period, whereas the values approaching one indicate high reproducibility. Specific gravity varied independently and exhibited moderate reproducibility throughout the pregnancy period (ICC = 0.42, 95% CI = 0.35 to 0.49). The correction of specific gravity decreased the temporal reproducibility of urinary BTHs, but improved the temporal reproducibility of urinary BTRs. For volume-based ICCs, urinary BTRs showed considerable within-subject variation over time where ICCs were low to moderate, with the highest ICC at 0.56 for TTR. The relatively high temporal reproducibility was observed for urinary BTHs with ICCs ranging from 0.42 to 0.85. Among these analytes, urinary 2-MeS-BTH (ICC = 0.85, 95% CI = 0.82 to 0.86) was the most stable BTs over pregnancy.

3.5. Predictors of urinary BTs among pregnant women

The linear mixed results in Table 4 showed that parity, household income, pregnancy occupational status, sampling season and menstrual cycle were significant predictors of the urinary BTs ($P < 0.05$). Maternal age was not associated with any urinary concentrations of BTs ($P > 0.05$). Parity was negatively associated with urinary concentrations of TTR ($P = 0.02$) but positively associated with that of 2-MeS-BTH ($P < 0.01$). Urinary concentrations of TTR ($P < 0.01$), 2-OH-



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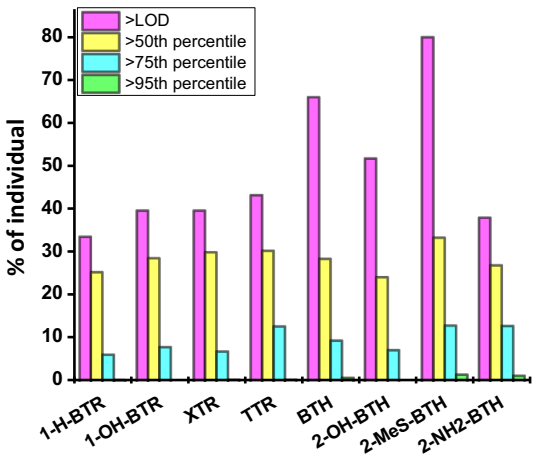

B

Fig. 2. Exposure patterns of BTs during pregnancy: (A) co-exposure pattern of all targeted BTs at each trimester (T1,T2,T3 for Trimester 1,2,3, respectively); (B) exposure to specific BTs across pregnancy.

Table 3
Intraclass correlation coefficients (ICCs) and 95% confidence intervals (95% CIs) for concentrations of urinary BTs during pregnancy.

Analytes	*n*	ICC (95% CI)	
		Unadjusted	SG-adjusted
1-H-BTR	288	0.12 (−0.07, 0.28)	0.14 (−0.05, 0.30)
1-OH-BTR	340	0.19 (0.03, 0.33)	0.45 (0.34, 0.54)
XTR	340	0.32 (0.18, 0.44)	0.38 (0.26, 0.48)
TTR	371	0.56 (0.48, 0.63)	0.57 (0.48, 0.65)
BTH	566	0.66 (0.60, 0.70)	0.49 (0.42, 0.55)
2-OH-BTH	444	0.42 (0.32, 0.51)	0.19 (0.07, 0.30)
2-MeS-BTH	686	0.85 (0.82, 0.86)	0.60 (0.55, 0.65)
2-NH_2-BTH	326	0.81 (0.77, 0.84)	0.36 (0.23, 0.47)
Specific gravity	856	0.42 (0.35, 0.49)	

The intra-class correlation coefficient calculation was only based on detectable samples.

BTH ($P < 0.01$) and 2-NH_2-BTH ($P = 0.02$) were higher among women from high income families (above 100,000 Chinese Yuan/year). Compared with those who were unemployed during pregnancy, the employed women exhibited higher levels of urinary 2-OH-BTH ($P < 0.01$), 2-MeS-BTH ($P = 0.05$) and 2-NH_2-BTH ($P = 0.03$). Across the four sampling seasons, the highest levels of urinary 1-H-BTR ($P < 0.01$), 2-OH-BTH ($P < 0.01$), 2-MeS-BTH ($P = 0.03$) and 2-NH_2-BTH ($P = 0.05$) were generally occurred in winter. Participants who suffered from irregular menstrual cycle had higher levels of urinary 2-MeS-BTH ($P < 0.01$) than those who did not.

4. Discussion

To the best of our knowledge, this is the first study administering repeated measures to characterize the exposure profiles, temporal variability and predictors of urinary BTs during pregnancy. The exposure of BTs was highly prevalent among pregnant women in Wuhan, one of the metropolitan cities in the central China. It was found that the exposure pattern of BTs likely to remain at low level and co-exposure to all the targeted compounds was infrequent during pregnancy. BTH was the predominant BTs in urine samples. Urinary concentrations of BTRs exhibited substantial within-subject variation during pregnancy. The relatively high temporal reliability was observed for all measured BTHs. Urinary concentrations of BTs were associated with the factors, such as parity, household income, pregnancy occupational status, sampling season and menstrual cycle. The findings are useful in reducing measurement error and eliminating confounding variables to optimize etiology studies.

4.1. Exposure profiles of BTs during pregnancy

In the present study, multiple urine samples collected at three different trimesters provided a more accurate estimation of gestational exposure to BTs. Reported studies on human exposure assessment of BTs were compiled in Table 5 to be compared with the findings of the present study. The exposure profiles of BTs in the current study were similar to that found in pregnant women from Tianjin (Li et al., 2018), with BTs widely detected at levels of the same magnitude (except for 2-OH-BTH). The occurrence of 1-OH-BTR observed among Chinese pregnant women may be partially due to direct exposure considering the wide presence of 1-OH-BTR (detection frequency > 96%, median: 2.28 ng/g) in indoor dust from China (Wang et al., 2013). In addition, a previous study theoretically proposed that the 1-OH-BTR in urine samples may originate from the metabolism of 1-H-BTR (Asimakopoulos et al., 2013b). Although the significant relationships between 1-H-BTR and its derivatives (e.g., 1-OH-BTR, TTR) were not

Table 4
Associations between ln-transformed concentrations of urinary BTs and demographic categories.

Characteristics	β estimates and 95% confidence intervals							
	1-H-BTR	1-OH-BTR	XTR	TTR	BTH	2-OH-BTH	2-MeS-BTH	2-NH_2-BTH
Age								
< 28	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
≥28	−0.01 (−0.23, 0.20)	0.01 (−0.19, 0.21)	0.02 (−0.15, 0.19)	0.02 (−0.11, 0.14)	−0.06 (−0.27, 0.15)	0.05 (−0.16, 0.25)	0.05 (−0.11, 0.21)	0.03 (−0.10, 0.16)
Parity								
1	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
≥2	0.01 (−0.29, 0.32)	−0.05 (−0.33, 0.23)	−0.05 (−0.30, 0.19)	**−0.15 (−0.28, −0.02)**	0.15 (−0.15, 0.44)	0.03 (−0.26, 0.32)	**0.34 (0.11, 0.57)**	−0.06 (−0.24, 0.13)
Household income (Yuan/year)								
Above 100,000	0.13 (−0.08, 0.35)	0.10 (−0.10, 0.29)	−0.13 (−0.31, 0.05)	**0.19 (0.07, 0.31)**	−0.18 (−0.39, 0.04)	**0.32 (0.09, 0.54)**	0.02 (−0.13, 0.17)	**0.15 (0.03, 0.27)**
50,000-100,000	−0.07 (−0.29, 0.16)	−0.13 (−0.33, 0.07)	−0.12 (−0.30, 0.06)	0.07 (−0.05, 0.19)	−0.04 (−0.26, 0.18)	0.11 (−0.11, 0.34)	−0.08 (−0.23, 0.07)	0.09 (−0.04, 0.21)
Below 50,000	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Pregnancy occupational status								
Unemployment	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Employment	0.11 (−0.15, 0.36)	0.13 (−0.10, 0.35)	−0.18 (−0.39, 0.03)	0.08 (−0.06, 0.22)	−0.15 (−0.40, 0.10)	**0.47 (0.21, 0.73)**	**0.17 (0.00, 0.35)**	**0.16 (0.02, 0.31)**
Sampling season								
Spring	0.10 (−0.12, 0.32)	−0.01 (−0.21, 0.18)	0.03 (−0.15, 0.21)	0.05 (−0.07, 0.17)	−0.15 (−0.36, 0.07)	0.13 (−0.08, 0.35)	**0.18 (0.03, 0.33)**	−0.05 (−0.18, 0.07)
Summer	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Fall	**0.36 (0.12, 0.59)**	0.05 (−0.16, 0.26)	−0.02 (−0.22, 0.17)	−0.10 (−0.23, 0.03)	−0.15 (−0.38, 0.07)	0.17 (−0.06, 0.40)	**0.18 (0.02, 0.34)**	0.06 (−0.07, 0.20)
Winter	**0.50 (0.28, 0.72)**	−0.01 (−0.21, 0.19)	0.01 (−0.17, 0.19)	0.11 (−0.01, 0.23)	−0.39 (−0.85, 0.08)	**0.59 (0.38, 0.81)**	**0.17 (0.02, 0.32)**	**0.13 (0.00, 0.25)**
Menstrual cycle								
Regular	Reference	Reference	Reference	Reference	Reference	Reference	Reference	Reference
Irregular	−0.05 (−0.37, 0.28)	0.03 (−0.26, 0.33)	−0.22 (−0.49, 0.04)	−0.03 (−0.21, 0.16)	0.06 (−0.26, 0.38)	−0.14 (−0.48, 0.19)	**0.31 (0.09, 0.53)**	−0.16 (−0.35, 0.02)

Table 5
Comparisons with other studies conducted on exposure assessment of BTs.

Study	Sampling year	Country	Population	Sample type	Unit	Sample size	Analytes										
							1-H-BTR	TTR	1-OH-BTR	XTR	5-Cl-H-BTR	ΣBTRs	BTH	2-NH_2-BTH	2-OH-BTH	2-MeS-BTH	2-SCNMeS-BTH	ΣBTHs
This study	2013–2015	China	Pregnant women	Urine	ng/mL	2568	0.32	0.11	0.77	0.12	0.026	0.80	1.6	0.052	0.46	0.33	0.032	2.4
Li et al., 2018	Oct.-Nov. 2015	China	Pregnant women	Urine	ng/mL	83	1.1	0.23	0.17	–	0.015	–	1.1	–	n.d	–	–	–
				Amniotic fluid	ng/mL	79	n.d	0.026	n.d	–	0.022	–	0.63	–	0.039	–	–	–
Asimakopoulos et al., 2016	May-14	Saudi Arabia	General	Urine	ng/mL	130	–	–	–	6.1	–	–	10	–	0.61	–	–	–
Asimakopoulos et al., 2013b	2010–2011	China	General	Urine	ng/mL	51	1.4	3.2	n.d	1.5	n.d	2.1	7.6	n.d	2.7	n.d	n.d	5.6
	2010–2011	India				46	2.0	n.d	n.d	2.1	n.d	2.1	5.4	1.3	7.5	n.d	n.d	4.5
	2010–2011	Japan				36	n.d	1.7	n.d	0.70	n.d	0.90	23	0.70	2.1	n.d	n.d	9.5
	2010–2011	Vietnam				25	5.4	n.d	n.d	1.1	n.d	1.5	10	n.d	2.2	n.d	n.d	11
	2010–2011	Korea				49	n.d	1.6	n.d	0.20	n.d	0.40	6.4	1.2	2.8	n.d	n.d	5.9
	2010–2011	U.S.				25	n.d	0.80	n.d	n.d	n.d	0.60	5.7	1.0	n.d	n.d	n.d	2.8
	March–April 2012	Greece				100	1.2	0.70	n.d	1.1	n.d	0.90	5.1	0.20	2.3	n.d	n.d	3.4
Wang et al., 2015	2003–2004	USA	General	Adipose fat	ng/g	20	n.d	1.6	n.d	0.73	n.d	–	n.d	n.d	5.5	n.d	–	–

Abbreviations: n.d. not detected –: not reported in the study.

found in urine samples (Li et al., 2018), a significant positive relationship between 1-H-BTR and TTR was reported in tap water, surface water and indoor air, indicating the co-exposure of the two contaminants or the degradation of 1-H-BTR into TTR (Wang et al., 2016; Wolschke et al., 2011; Xue et al., 2016). Therefore, the possibility of formation from 1-H-BTR to its derivatives in human bodies cannot be ruled out (Wang et al., 2016; Wolschke et al., 2011; Xue et al., 2016; Asimakopoulos et al., 2013b). Future studies are warranted to elucidate the metabolic mechanism of BTs in human body.

Compared to BTRs (ΣBTRs GM: 0.80 ng/mL), BTHs (ΣBTHs GM: 2.4 ng/mL) exhibited higher abundance in human body with the compound BTH playing the predominant role (GM: 1.6 ng/mL). This phenomenon may be explained by the dominance of BTHs observed in indoor dust (ΣBTRs GM:19.2 ng/g; ΣBTHs GM: 857 ng/g) and tap water (ΣBTRs GM:15.6 ng/L; ΣBTHs GM: 406 ng/L) from China (Wang et al., 2013; Wang et al., 2016). Although heating and boiling can efficiently remove 80% of the BTH in tap water, water consumption was estimated to account for 12.3% of the total BTH intake (Wang et al., 2016). Consistent with previous human biomonitoring studies (Li et al., 2018; Asimakopoulos et al., 2013b), urinary 5-Cl-H-BTR (9.3%; 0.03 ng/mL) and 2-SCNMeS-BTH (1.4%; 0.04 ng/mL) were occasionally detected here. The low detection frequency of 5-Cl-H-BTR is not surprising as the chemical also exhibited low abundance in indoor dust (72.7%) (Wang et al., 2013), indoor air (1.2%) (Xue et al., 2016) and tap water (18.6%) (Wang et al., 2016) with a median concentration of 1.27 ng/g, 0.13 ng/m^3 and < LOD, respectively. Similarly, 2-SCNMeS-BTH was occasionally detected in indoor dust samples with detection frequency of 9.1% (Wang et al., 2013). In addition, it was reported that 2-SCNMeS-BTH was unstable and prone to undergo hydrolysis and/or photolysis to form the more stable products such as 2-mercaptobenzothiazole, 2-MeS-BTH, 2-OH-BTH and BTH in aquatic system (Brownlee et al., 1992). Therefore, the low abundance of 5-Cl-H-BTR and 2-SCNMeS-BTH in the environment may be responsible for their absence in human biomonitoring studies.

As for the correlation analysis of urinary BTs, we observed that correlations among BTHs (r = 0.02–0.14) were higher than that among BTRs (r = 0.04–0.39), indicating the shared exposure sources (Avagyan et al., 2015; Trabalon et al., 2017) or common metabolic pathway among BTHs (Brownlee et al., 1992). Additionally, there were some moderate positive correlations between BTRs and BTHs (e.g., r = 0.47 for TTR and 2-MeS-BTH, P < 0.01). We speculate that the correlations among chemicals from different classes could be due to the consumption of products that contain both BTRs and BTHs. However, this interpretation should be treated with caution because we did not have information about the personal care product usage.

As for the exposure pattern analysis, it was found that co-exposure to all the targeted compounds was infrequent among Chinese pregnant women and the pro-longed exposure to a specific compound at low level during pregnancy was frequently observed. The lack of comparable studies in this regard suggested that more efforts are needed in future to investigate the health effect of pro-longed exposure to BTs at low level.

4.2. ICCs and implications for sampling scheme in etiological studies

We estimated ICCs based on the repeated measurements to evaluate the temporal variability of urinary BTs during pregnancy. An ICC of 0.40 was regarded as a threshold for sufficient reproducibility of a biomarker used in epidemiology studies (Rosner, 2000). There was a moderate reproducibility of specific gravity with an ICC of 0.42 which showed good accordance with a previous study conducted on women during pregnancy (Ferguson et al., 2014). The physiological changes among participants like the increased kidney size and the precipitous drop in the renal blood flow rate in late pregnancy may be responsible for the variation (Cheung and Lafayette, 2013).

In the volume-based ICC analysis, urinary concentration of 2-MeS-

BTH showed high temporal reproducibility with an ICC of 0.85 (95% CI = 0.82 to 0.86), indicating that exposure might come from sources that are fairly consistent over time. We further speculate that a single spot urine sample has the potential to accurately reflect exposure level of 2-MeS-BTH during pregnancy. However, the substantial variation was observed for urinary concentrations of 1-H-BTR (ICC = 0.12, 95% CI = −0.07 to 0.28), 1-OH-BTR (ICC = 0.19, 95% CI = 0.03 to 0.33) and XTR (ICC = 0.32, 95% CI = 0.18 to 0.44) during pregnancy. This indicated that exposure misclassification might occur when relying on one spot urine sample to represent the exposure level over a period of time. Therefore, collection and analysis of multiple samples during pregnancy are warranted in gestational exposure-health effect studies to reduce exposure measurement error and to explore susceptible windows of adverse health outcome. But this would substantially increase the costs and participants burden, which may affect the participation and follow-up rates (Galea and Tracy, 2007). Recording the time of urine collection and the time since the last void has been proposed as one recommended remedy (Preau et al., 2010). Another solution would be to categorize subjects into low- and high- exposure groups. Regardless of whether multiple samples are to be collected in future studies, our results provided valuable information for researchers of etiological studies to optimize sampling strategies to manage the risks of misclassifying exposure and biasing the health effects.

In this study, urinary concentrations of BTHs exhibited generally higher reproducibility (ICCs: 0.42–0.85) than BTRs (ICCs: 0.12–0.56). The different variability patterns between BTHs and BTRs may be attributed to their distinct exposure, metabolism, elimination pathways. Notably, adjusting for specific gravity improved the reproducibility of urinary BTRs, but reduced that of BTHs. According to the data on the C18 column (Fig. S1), BTRs had shorter retention time than BTHs (1-OH-BTR < 2-NH_2-BTH < 1-H-BTR < TTR < 5-Cl-H-BTR < XTR = 2-OH-BTH < BTH < 2-MeS-BTH < 2-SCNMeS-BTH), indicating the increased polarity of BTRs. Therefore, using specific gravity to adjust for the urine dilution may improve the reproducibility of urinary polar biomarkers, such as BTRs. Caution is required in the generalization of our findings to other populations given that our subjects were pregnant women who were different from the general population (Cheung and Lafayette, 2013).

4.3. Predictors of urinary BTs during pregnancy

The demographic factors in this study were investigated to explore the associations of these factors with urinary concentrations of BTs. Parity was found to be negatively associated with urinary concentration of TTR. Previous studies reported that TTR was frequently detected in amniotic fluid (86.1%) (Li et al., 2018) and adipose fat (95.0%) (Wang et al., 2015) with GM concentrations of 0.026 ng/mL and 1.55 ng/g, respectively. We speculate that the partition to amniotic fluid or elimination through placenta after delivery may account for the lower abundance of TTR among women who were multiparous. However, urinary concentrations of 2-MeS-BTH were higher among multiparous pregnant women. Similar results were observed in a previous study where the urinary concentration of phthalate metabolites were found to be positively associated with the parity (Gao et al., 2017). However, the reason was not well understood.

Pregnant women from high income families (above 100,000 Chinese Yuan/year) tended to have higher levels of urinary TTR ($P < 0.01$), 2-OH-BTH ($P < 0.01$) and 2-NH_2-BTH ($P = 0.02$). Similarly, one study conducted on tap water in China observed the increased concentration of BTRs with elevated economic status, in which the GM concentrations of BTRs in tap water from the least, middle, and most developed zone were 10.9 ng/L, 16.5 ng/L and 30.7 ng/L, respectively (Wang et al., 2016). BTs are widely used in corrosion inhibitors, ultraviolet (UV) stabilizers, deicing fluids, dishwater detergents and fungicides (Richardson and Ternes, 2018; Liao et al., 2018). It was reported that organic UV filter concentrations in indoor dust increased with annual income (Ao et al., 2018). Therefore, household income as an indicator of social-economic status, might be associated with the usage of personal care products that contain BTs (Wu et al., 2010).

Urinary concentrations of 2-OH-BTH ($P < 0.01$), 2-MeS-BTH ($P = 0.05$) and 2-NH_2-BTH ($P = 0.03$) were significantly higher among women who were employed during pregnancy, suggesting the importance of occupational source for daily exposure. In our study, sampling season was associated with the urinary concentrations of 1-H-BTR ($P < 0.01$), 2-OH-BTH ($P < 0.01$), 2-MeS-BTH ($P = 0.03$) and 2-NH_2-BTH ($P = 0.05$), with the lowest exposure level observed in summer and highest exposure level that generally occurred during winter. A similar seasonal variation of BTs was reported in water from Yangtze River, China, where the benzotriazoles in dry season (November) were significantly higher than those in wet season (July) (Yao et al., 2018). This phenomenon may be due to the higher consumption of antifreeze fluid that contains BTs during winter months (Seeland et al., 2012).

Interestingly, the participants who suffered from irregular menstruation cycle (variation ≥7 d) had significantly higher concentration of urinary 2-MeS-BTH. As urinary concentration of 2-MeS-BTH exhibited high temporal reproducibility (ICC = 0.85, 95% CI = 0.82 to 0.86) for a given time period of several months (time interval of sample collection), we assumed that urinary concentration of 2-MeS-BTH during pregnancy could reflect the exposure level of 2-MeS-BTH before pregnancy. Based on this assumption, we speculate that the higher exposure of 2-MeS-BTH might increase the risk of irregular menstruation. Although there is no direct evidence suggesting that exposure to 2-MeS-BTH can contribute to irregular menstrual cycle, previous study indicated that BTHs could disrupt hypothalamic–pituitary–thyroid axis (HPT) by inhibiting the synthesis of thyroid peroxidase and the release of thyroxine (T4) (Hornung et al., 2015). Since the hypothalamic–pituitary–ovarian axis (HPO) and the HPT act as a unified system in human body (Doufas and Mastorakos, 2000), heterostasis of the thyroid hormones caused by BTHs derivative could be one of the factors leading the irregular menstruation.

4.4. Strengths and limitations of the study

One of the strengths of the current study is that it fills a gap in understanding the exposure profiles, temporal variability and predictors of urinary BTs biomarkers among pregnant women in China. Multiple urine samples collected on the same subject at three different trimesters allowed a more accurate estimation of the BTs exposure and could serve as the benchmark for future work. We reported the first study on ICCs to evaluate the variability of urinary BTs over the pregnancy and provided insights into how to reduce measurement errors for sampling schemes. Furthermore, demographic factors were investigated to identify the predictors of exposure. From a public health perspective, this information, coupled with data from other biomonitoring studies may help inform researchers and policy makers about the sources of BTs exposure among pregnant women and eliminate potential confounding variables in etiology studies. Notably, BTH derivatives as an important drug intermediate have been widely used in pharmacological agents (Gill et al., 2015; Khan et al., 2016). However, given the constraints of the data collection, little was known about the dietary intake, personal care products and pharmacological usage of the participants during pregnancy, which might limit our ability to identify other factors that may influence exposure patterns.

5. Conclusions

The results from this study showed that exposure to BTs was highly prevalent among pregnant women in China. Co-exposure to all the targeted compounds was infrequent and the exposure to specific compound was constant at low level during pregnancy. Urinary concentrations of BTRs exhibited substantial within-subject variation

during pregnancy. In etiology studies that aim at characterizing the dose-response effects, adopting the study design based on multiple urine samples collected throughout the exposure window is highly recommended to limit exposure misclassification and to reduce the bias in health effects. Factors that were associated with urinary concentrations of BTs such as parity, sampling season, household income and pregnancy occupational status, provided valuable information on tracing exposure sources and should be taken into consideration when exploring the associations between gestational exposure to BTs and birth outcomes. Future studies should strive to identify the possible metabolic pathways and evaluate the potential health effects of pro-longed exposure to BTs at low level, especially among susceptible populations.

Disclosures

The authors declare no competing financial interest.

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Appendix A. Supplementary data

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