# URINANRY BROMOPHENOLS PHASE II CONJUGATES: A NOVEL HUMAN EXPOSURE BIOMARKS FOR PBDEs

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## Introduction

Even though their uses have already been heavily restricted in many parts of the world, polybrominated diphenyl ethers (PBDEs) are still widely presented as flame retardant (FR) additives in polymers, electric appliances and textiles. PBDEs with 4 to 6 bromine substituents have already been formally listed as POPs by the Stockholm Convention in 2009 because of the solid evidences indicating their presence in the global ecosystem[1]. To monitor human exposure to PBDEs, the most frequently adopted approach is direct quantification of selected BDE congeners and their hydroxy- / methoxy- conjugates in human tissues, for example: human plasma or serum[2,3], breast milk[4], and adipose tissues[5]. However, samplings of these tissues are intrusive operations which are difficult to achieve in large-scale population-wide or nation-wide surveys. Sampling of breast milk can be considered a non-intrusive process, but samples are only restricted to lactating women of a narrow range of ages. It may not be able to truly reflect the level of population exposure to contaminations. Thus, to reliably assess the population exposure of PBDEs, a non-intrusive, representative and convenient sampling and analytical protocol is needed. Sampling of human urine is a truly non-intrusive process and human urine is much easier to obtain from voluntary donors, for large-scale exposure surveys, than any other human tissues. There are already numerous studies that used urinary metabolites as exposure biomarkers for different types of contaminants[6,7,8,9]. It would be ideal if the presence of PBDE metabolites / PBDE congeners in human urine can be used as biomarkers for human exposure to PBDEs. This would greatly facilitate nationwide and international comparisons and public health risk assessments of PBDE exposure. Pharmacokinetic and toxicokinetic studies of BDE-47 and BDE-99 in mammalian animal models have already identified the presence of Phase II metabolites, mainly dibromophenols (DBPs) and tribromophenol (TBPs) glucuronide and sulfate conjugates, in urine samples. These bromophenol (BP) metabolites were believed to be originated from the cleavage of the ether linkage between the two aromatic rings of the BDE congeners. It is hypothesized that these ether-link cleaved Phase II metabolites of BDE congeners are also presented in human urine and their levels can be used to correlate the extent of exposure to PBDEs. If this hypothesis is correct, a more reliable population exposure assessment for PBDEs can be developed via the measurement of the glucuronide and sulfate conjugates of BPs in human urine. To examine this hypothesis, we synthesized a number

of glucuronide and sulfate conjugates of bromophenols and studied the correlation between levels of these compounds in human urine and those of the parent BDE congeners in human blood plasma.

**Materials and Methods**

All paralleled human blood and urine samples were collected randomly from volunteers by certified medical personnel in Hong Kong. Both plasma and urine samples were kept frozen at -80 $^{o}$C until analysis. A slightly modified extraction and clean-up method from the literature[12,13] was adopted for analyte extraction from human plasma samples. Authentic standards of the glucuronide and sulfate conjugates of 2,4-dibromophenol and 2,4,6-tribromophenol were synthesized and purified by in-house procedures. These four BP conjugates were fully characterized by high-resolution mass spectrometry, elemental analysis and $^{1}$H NMR spectroscopy. Their purity was constantly checked by LC-MS with triple quadrupole tandem mass spectrometer. 17 PBDE congeners were studied in this study including mono- to deca-bromo substituted, 15 Methoxy- and 18 hydroxyl-BDE congeners were also quantified in human plasma sample and summarize in the following table. 2,4-dibromphenol, 2,4,5-tribromophenol and 2,4,6-tribromophenol were analysis in this studied.

| BDE Congener | | Hydroxy-BDEs | | Methoxy-BDEs | |
|---|---|---|---|---|---|
| BDE-3 | BDE-15 | 2'-OH-BDE-7 | 3'-OH-BDE-7 | 4'-OMe-BDE-17 | 6'-OMe-BDE-17 |
| BDE-28 | BDE-47 | 4'-OH-BDE-17 | 2'-OH-BDE-28 | 2'-OMe-BDE-28 | 5-OMe-BDE-47 |
| BDE-66 | BDE-85 | 4-OH BDE-42 | 3-OH BDE-47 | 6-OMe-BDE-47 | 4'-OMe-BDE-49 |
| BDE-99 | BDE-100 | 6-OH BDE-47 | 4'-OH-BDE-49 | 2'-OMe-BDE-68 | 6-OMe-BDE-85 |
| BDE-153 | BDE-154 | 2'-OH-BDE-66 | 2'-OH-BDE-68 | 3-OMe-BDE-90 | 6-OMe-BDE-90 |
| BDE-183 | BDE-184 | 6-OH-BDE-85 | 4-OH-BDE-90 | 3-OMe-BDE-100 | 2-OMe-BDE-123 |
| BDE-197 | BDE-202 | 6-OH-BDE-90 | 5'-OH-BDE-99 | 6-OMe-BDE-137 | |
| BDE207 | BDE-208 | 6'-OH-BDE-99 | 3-OH-BDE-100 | | |
| BDE-209 | | 2-OH-BDE-123 | 6-OH-BDE-137 | | |

Table 1. BDEs, OMe-BDEs and OH-BDEs congeners analyzed in human plasma samples

Solid-phase extraction was adopted for the extraction of BPs glucuronide and sulfate conjugates in human urine. Briefly, 5 mL of human urine sample was partitioned with 3 × 10 mL of ethyl acetate. The combined organic solution was evaporated into dryness under a gentle stream of nitrogen. The residue was dissolved in Milli-Q water (10 mL) and sodium acetate solution (5 mL, 2 M). the resultant solution was applied at a flow rate of 1 drop / second to a previously conditioned Oasis® WAX cartridge. The loaded WAX cartridge was then washed with 5 mL of 2M sodium acetate buffer and 5 mL of methanol. The glucuronide fraction was eluted by 4 mL of formic acid: methanol (1:9, v/v) and the sulfate fraction was eluted by 4 mL of ammonium hydroxide : $H_2O$ : methanol (1:2:17, v/v/v). The eluates were evaporated to around 100 μL under a gentle stream of nitrogen, and

200 μL of 500 ng/mL $^{13}C_6$-2,4-dibromphenol was added as an internal standard. Creatinine determination was carried out by a kinetic colorimetric assay based on the modified Jaffe method.[14] Spearman Rank Order test was employed for the correlation analysis using the statistical software SPSS 16 or SigmaStat 3.5.

**Results and Discussion**

PBDEs were detected in all the 100 human plasma samples with Σ[PBDE] ranging from 0.01 – 23.48 ng/g-lipid. The Σ[PBDE] obtained in our study is quite compared to previous studies from other parts of the world[15,16,17,18,19]. Among the congeners detected in our samples, BDE-47, -99 and -209 were the three most predominant congeners detected. OH-BDEs and BPs were also detected in human blood samples. 6-OH BDE-47 and 5'-OH BDE-99 were the two most predominated hydroxylated BDE species, which showed a mean concentration of 0.58 and 0.22 ng/g-lipid, respectively. Three different BPs were detected in 80% of the blood plasma samples and their mean levels ranged from 0.53 to 1.85 ng/g-lipid. OMe-BDEs were also identified and quantified in human blood samples. To the best of our knowledge, our study is the first to report OMe-BDEs in human blood samples. 6-OMe-BDE-47 and 4-OMe-BDE-17 were the two most predominated methoxylated BDE species. Mean concentrations of these two species in human plasma were 0.88 and 0.73 ng/g-lipid, respectively. All four types of BP conjugates were detected in human urine samples, ranged from 0.08 to 106.49 μg/g-creatinine. 2,4,6-Tribromophenyl (2,4,6-TBP) conjugates were more frequently detected (around 90% of all the human urine samples) than the 2,4-dibromophenol (2,4-DBP) conjugates. The detection frequencies of 2,4-DBP conjugates were around 70% of all the samples.

Multiple linear regression models were used to estimate of the beta coefficients of the BP conjugates with other variables. In the multiple linear regression analyses, we included, as the dependent variable, the sum of total natural-log transformed BP conjugated metabolites in urine, and, as predictors, ΣPBDEs, ΣOMe-PBDEs, ΣOH-PBDEs and ΣBPs. In this model, the adjusted R-square ($R^2$) was 0.358, which indicated the proportion of variability in the dependent value accounted by this model. We also observed that ΣPBDEs and ΣBPs have significantly affected the prediction of the dependent, with standardized coefficients (β) of 0.378 and 0.335 respectively (significant level: ΣPBDEs, < 0.001 and ΣBPs, 0.004). Then we selected the ΣPBDEs, ΣOMe-PBDEs, ΣOH-PBDEs and 2,4-DBP / 2,4,6-TBP with same structural analogues to be our determinants of the multiple linear regression model and this has led to a much better prediction results. These revised models gave outstanding adjusted $R^2$ of 0.828 (Σ2,4-dibromo-BDE) and 0.749 (Σ2,4,6-tribromo-BDE). They also gave a better standardize coefficients, with β for Σ2,4-dibromo-BDE and Σ2,4,6-tribromo-BDE being 0.822 and 0.856 respectively. We also found no multicollinearity among variables, as the variance inflation factor did not excess 2 in any of our models. Our result shows that the a strong relationship between BP glucuronide and sulfate conjugates and ΣPBDEs (especially for Σ2,4-dibromo-BDE and Σ2,4,6-tribromo-BDE) outweighs

ΣOMe-PBDEs, ΣOH-PBDEs and ΣBPs. Pearsons product moment correlation was used to test the correlation among natural-log transformed ΣBP conjugated metabolites and ΣPBDEs, ΣOMe-PBDEs, ΣOH-PBDEs and ΣBPs when appropriate. We found a moderate relationship, r = 0.458, between ΣPBDEs and ΣBPs conjugates in total human urine samples. Generally speaking, the concentration of PBDEs in human plasma increases with increasing urinary BPs glucuronide and sulfate conjugates. We further studied analytes with same structure, e.g. Σ2,4-dibromo-BDE (Sum of BDE-28, BDE-47, BDE-66, BDE-85, BDE-99, BDE-100) / Σ2,4,6-tribromo-BDE (Sum of BDE-100, BDE-154. BDE-184) in human plasma and Σ2,4-DBP conjugates / Σ2,4,6-TBP conjugates in human urine and they showed excellent correlation (r = 0.911 and r = 0.982 respectively). These good correlations suggest that the BP conjugates in human urines can be adopted as exposure markers for PBDE, especially those of Penta- and Octa-BDEs, in human.

**Summary**

This is the first study to show significant relationships between plasma PBDEs and urinary BPs conjugates in human. This result is important because it confirms BPs glucuronide and sulfate conjugates can be used as exposure markers for the truly non-intrusive assessment of population exposure to PBDEs.

**References**


1. Alaee M., Arias, P., Sjodin, A., Bergman, A.. 2003. *Environ. Int.*, 29, 683 – 689
2. Wan Y., Choi K., Kim S., Ji K., Chang H., Wiseman S., Jones P. D., Khim J. S., Park S., Park J., Lam M. H. W., Giesy J. P., 2010. *Environ. Sci. Technol.*, 44, 5233 – 5239
3. Kang C. S., Lee H. S., Kim S. K., Lee K. T., Lee J. S., Park P. S., Yun S. H., Kannan K., Yoo Y. W., Ha J. Y., Lee S. W. 2010. *Chemosphere*, 80,116 – 122
4. Daniels J. L., Pan I. J., Jones R., Anderson S.. Patterson D. G. Jr., Needham L., Sjödin A.,. 2010 *Environ Health Perspect.*, 118:155 – 160
5. Kalantzi O. I., Brown F. R., Caleffi M., Goth-Goldstein R., Petreas M., 2009. *Environ. Int.*, 35, 113 – 117
6. Chen Z., Maartens F., Vega H., Kunene S., Gumede J., Krieger R. I.. 2009. *Int. J. Toxico.*, 26, 528 – 533
7. Kile M. L., Hoffman E., Hsueh Y. M., Afroz S., Quamruzzaman Q., Rahman M. Mahiuddin, G., Ryan, L., Christiani, D. C. 2009. *Environ. Health. Perspect.*, 117, 455 – 460
8. Lu C., Barr D. B., Pearson M., Bartell S., Bravo R. 2006. *Environ. Health Perspect.*, 114, 1419 – 1423
9. Talaska G., Underwood P., Maier A., Lewtas J., Rothman N., Jaeger M. 1996. *Environ. Health Perspect.*, 104, 901 – 906
10. Chen L. J., Lebetkin E. H., Sanders J. M., Burka L. T. 2006. *Xenobiotica*, 36, 515 – 534
11. Sanders J. M., Chen L. J., Lebetkin E. H., Burka L. T. 2006. *Xenobiotica*, 36, 103 – 117.
12. Hovender L., Athahasiadou M., Asplund L., Jensen S., Wehler E. K. 2000. *J. Anal. Toxicol.* 24, 696 – 703
13. Qiu X., Bigsby R. M., Hites R. A. 2009. *Environ. Health Perspect.*, 117, 93 – 98.
14. Bartels H., Bohmer M., Heierli C. 1972. *Chin. Chim. Acta.* 37,193 – 197
15. Zhu L., Ma B., Hites R. A., 2009. *Environ. Sci. Technol.*, 43, 6963 – 6968
16. Harrad S., Porter L. 2007. *Chemosphere*, 66, 2019 – 2023
17. Lee H. S., Ikonomou M. G., Park H., Beak S. Y., Chang Y. S. 2007. *Cemosphere*, 67, 489 – 497
18. Weiss J., Wallin E., Axmon A., Jönsson B. A. G., Åkesson H., Janák K., Hagmar L., Bergman Å. 2006. *Environ. Sci. Technol.*, 40, 6282 – 6289
19. Meijer L., Weiss J., van Velzen M., Brouwer A., Bergman Å., Sauer P. J. J. 2008. *Environ. Sci. Technol.*, 42, 3428 – 3433