A metabonomic method for profiling of metabolic changes in Hexabromocyclododecane and Tetrabromobisphenol A induced rat serum

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Abstract

This work characterizes the metabolism disorders of rat induced by TBBPA or HBCD in a Sprague Dawley rat model with two dosage of 30mg/kg and 300mg/kg. A seven-day long continuous administration was performed in male rats and sacrificed on Day 8 for serum collecting in this experiment. An integrated analytical approach based on Ultra performance liquid chromatography coupled with quatropde-time of flight mass spectrometer (UPLC-Q-TOF-MS) was developed to map the metabolic response in serum. LC-MS spectra of the serum were analyzed via pattern recognition using partial least squares discriminant analysis (PLS-DA). The PLS-DA score plot presented distinct separation between control group and dosed groups of TBBPA or HBCD. It was demonstrated that the metabolic patterns of the rats have been disturbed after oral dosed. The metabonomic approach based on LC–MS indicated several kinds of endogenous metabolites as biomarkers in serum associated with metabolic disorders induced by TBBPA or HBCD. The present study proves the great potential of LC–MS based metabonomics in mapping metabolic response for toxicology.

Introduction

Hexabromocyclododecane (HBCD) and tetrabromobisphenol A (TBBPA) are two Brominated flame retardants (BFRs) currently in use. In commercial HBCD, γ-HBCD is the main component, consisting 75%-89% of the total weight (α-HBCD, 10-15%; β-HBCD, 0.5%-12%). The production capacity of TBBPA and HBCD in China are about 18000 and 7500 metric tons in 2007 respectively[1]. Studies indicated that BFRs are bioaccumulative and persistent compounds, thus they should be regarded as persistent organic pollutants (POPs)[2]. However, there were relatively few studies reporting the toxic effect of HBCD and TBBPA to animal or human being.

Metabonomic research using LC–MS as an analytical platform has attracted a great deal of interest in toxicological research, as well as in biomarker discovery. 2.3 In recent years, the development of higher performance LC systems in separation technology has provided a promising high throughput platform to separate thousands of metabolites in biological samples over a shorter cycle time with increased resolution. Such a development has expedited method development and validation in LC–MS-based metabonomic research. 4 In this paper, metabolism disorders induced by TBBPA or HBCD in a rat model with different dosage of intoxication were studied by metabonomics in the basis of LC-MS.

Materials and Methods

Chemicals and reagents HPLC grade acetonitrile was purchased from Fisher Scientific. Formic acid, ammonium acetate in HPLC grade, HBCD and TBBPA in analytical grade, and sodium carboxymethyl- cellulose

were purchased from Sigma Co.

Animals and treatments Male Sprague Dawley rats (n = 40) weighing 200–250 g from Academy of Military Medical Sciences were used in this study and acclimatized for oneweek in the standard animal house prior to administration. The light cycle was consisted of 12 h light and 12 h darkness at a temperature of 25±2 ◦C with the humidity from 45% to 65%. The animals had free access to certified standard diet and drinking water and were divided randomly into 5 groups include control group, TBBPA high and low dosed group (30 and 300mg/kg/day), HBCD high and low dosed group (30 and 300mg/kg/day), each group containing eight animals. Rats were given daily TBBPA and HBCD which mixed with 0.5%CMC water solution for seven days and sacrificed on the eighth day for serum collecting. Collected serum samples were immediately used for pre-treatment.

Serum sample preparation The blood samples were centrifuged at 3000rpm for 10min to separate serum. 400μL of serum was mixed with 800μL of acetonitrile for protein-precipitation, after vortex for 2min; the mixture was centrifuged at 15000rpm for 10min, 400μL of the supernatant was transferred to a LC vial for LC-MS analysis.

LC condition Chromatography was performed on a Waters Acquity UPLC™ system (Waters, Manchester, UK) using an Acquity UPLC BEH C18 column (1.7μm, 2.1mm×100mm; Waters). The column oven temperature was maintained at 40 ◦C and the autosampler temperature maintained at 4◦C. The flow rate was 0.3mL/min and injection volume was 10μl using partial loop mode. When the MS was performed at positive ion electrospray mode, the mobile phases were (A) 0.1% formic acid in water, and (B) acetonitrile with 0.1% formic acid; while under negative mode, the mobile phases were 2mM ammonium acetate in water, and (B) acetonitrile. The gradient program was list in table 1.

Table 1 gradient program for ESI(+) and ESI(－)

ESI+			ESI -		
Time	A%	B%	Time	A%	B%
0	98	2	0	95	5
1	98	2	1	95	5
3	75	25	3	25	75
11	0	100	9	2	98
13	98	2	11	2	98
16	98	2	16	95	5

Mass spectrometry parameters Mass spectrometry was performed by using a Waters Micromass Q-TOF Premier™ (Waters, Manchester, UK) operating in both positive and negative ion electrospray modes. The source temperature was set at 100 ◦C while the desolvation temperature was at 300◦C, with nebulisation gas set to 500L/h. The capillary voltages were set at 2.8 kV and 3.0kV respectively in positive and negative ionisation mode. A LockSpray™ interface was used to ensure mass accuracy. For this, leucine-enkephalin (m/z556.2771 or 554.2615 in positive and negative ionisation modes respectively) was infused at a concentration of 200 pg/μL at a flow rate of 30μl/min. Data were collected in centroid mode over the range 65–1000m/z with an acquisition rate of 0.2s, interscan delay of 0.02s, with dynamic range enhancement activated.

Data analysis The acquired data were analysed and visualised using Waters MassLynx (Ver 4.1) and Waters MarkerLynx (Ver 4.1). The noise elimination level was set at 6, with 15 masses per retention time collected. Background ions from associated blank injections were excluded. The mass and retention time windows were set at 0.05 Da and 0.2min respectively. Multivariate analysis of the processed data were performed and visualised

using SIMCA-P + (Ver 21.0,Umetrics, Sweden). All the variables were set to Pareto scaling. The dataset was then "autofit" and visualised by partial least squares discriminant analysis (PLS-DA) to discriminate any differences between groups.

Results and Discussion

Generally, hundreds of peaks were identified by MassLynx from each LC–MS run. Typical total ion chromatograms generated from the different dosed group and control group are shown in Fig. 1.The detected peaks in extracted ion chromatogram were aligned mainly based on their retention time similarity. Any missing data points of a peak were filled in by integrating the raw aligned LC–MS files according to the second-derivative Gaussian function[5]. Then each variable in the data set was normalized to be proportional to the summation of total excreted variables in the same sample. Normalized data set was fed to multivariate analysis to characterize the metabolic pattern of rat serum.

PLS-DA was performed for the acquired metabonomic data table. Which was a classic supervised method (prior knowledge concerning groups or tendencies within the data sets was necessary) for pattern recognition. The PLS-DA score plots of control group and dosed group were shown in Fig 2 and Fig 3. The score plot presented distinct separation between control group and dosed groups of TBBPA or HBCD, suggesting the metabolic patterns of the rat serum have been disturbed after seven-day oral dosed. From score plot of TBBPA dosed group and control group, it can be observed that the separation between high dosed group and low dosed group was clear, thus suggesting a positive correlation of dose dependent effect. However, in the score plot of HBCD, sample of high dosed group and low dosed group was overlap under ESI+ mode, indicating that the metabolic patterns might not be further deteriorated by increasing HBCD dosage.

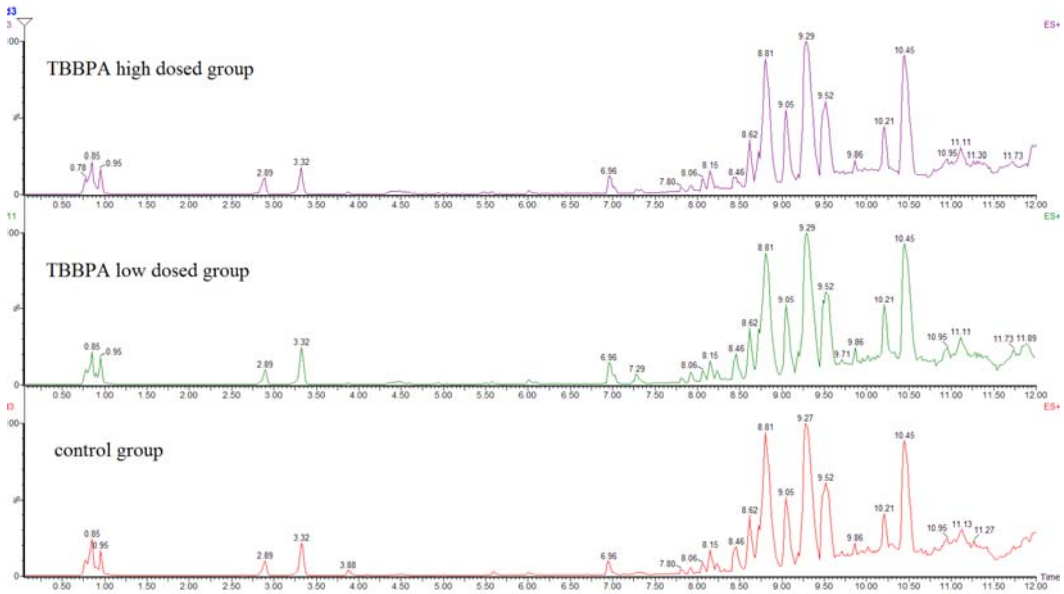

Fig. 1 Typical total ion chromatograms showing serum profiles analysed under ESI+ mode

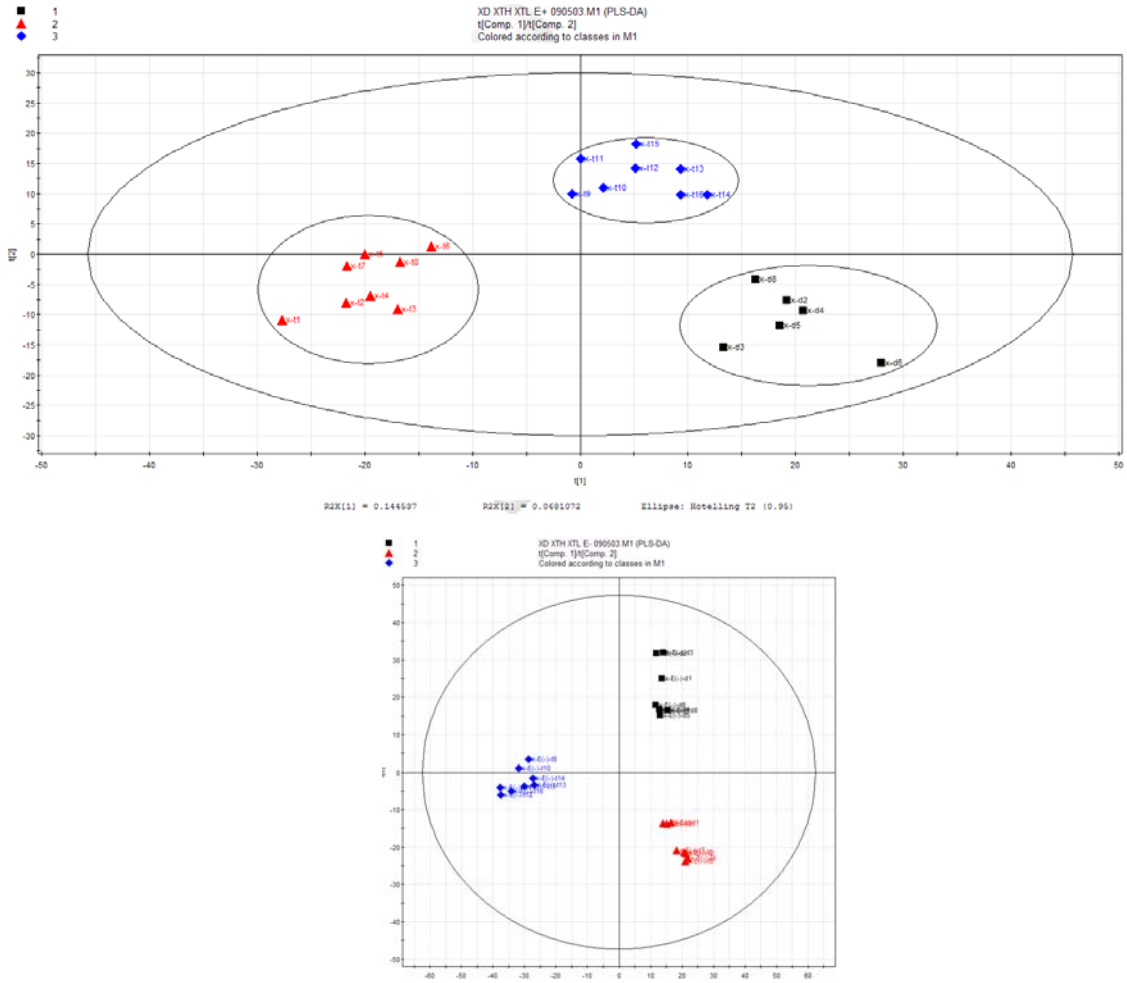

Fig.2 Score plot of PLS-DA performance. Serum samples of control group and dosed group of TBBPA.（■: control group，◆: low dosed group，▲high dosed group，Upper: ESI+ mode, Nether: ESI- mode）

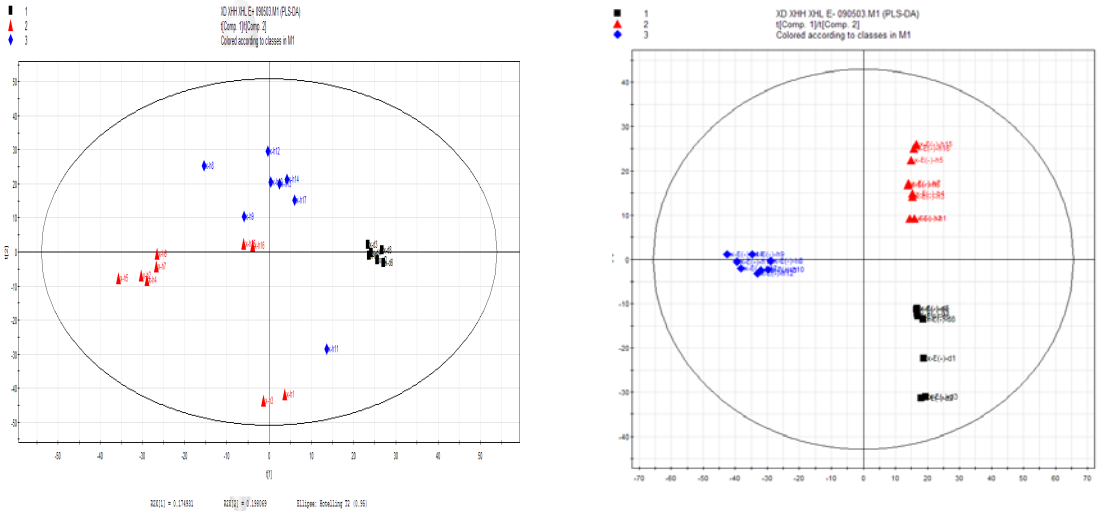

Fig. 3 Score plot of PLS-DA performance. Serum samples of control group and dosed group of HBCD .（■：control group，◆：low dosed group，▲high dosed group，Left: ESI+ mode, Right: ESI-mode）

Identification of the endogenous metabolites The main objective of metabonomic analysis is not only to detect variations in chromatographic fingerprints, but also to identify the compounds that are responsible for these variations. Some metabolites were screened from the loading plot for further identification (Fig. 4). Without available authentic references, full scan mass spectra of these metabolites addressing their characteristic masses were interpreted using available biochemical databases, such as Metlin, SciFinder, and KEGG and so on. Meanwhile, CID fragmentation patterns were combined to deduce possible structures of those potential biomarkers. According to the database, those biomarkers should be some kinds of amino acid, steroid, glucoside and phospholipid etc. For example, level of a component (m/z415.3532 at 8.53min) was found to have distinct change tendency between control group and HBCD dosed group (Fig. 5). Thus, it was considered as a potential biomarker. With Metlin database searching[6], it should be a steroid. However, since there are too many responses for a certain mass in database search and LC–MS fragmentation characteristics are different because of various operation conditions, the identified biomarkers were ambiguous.

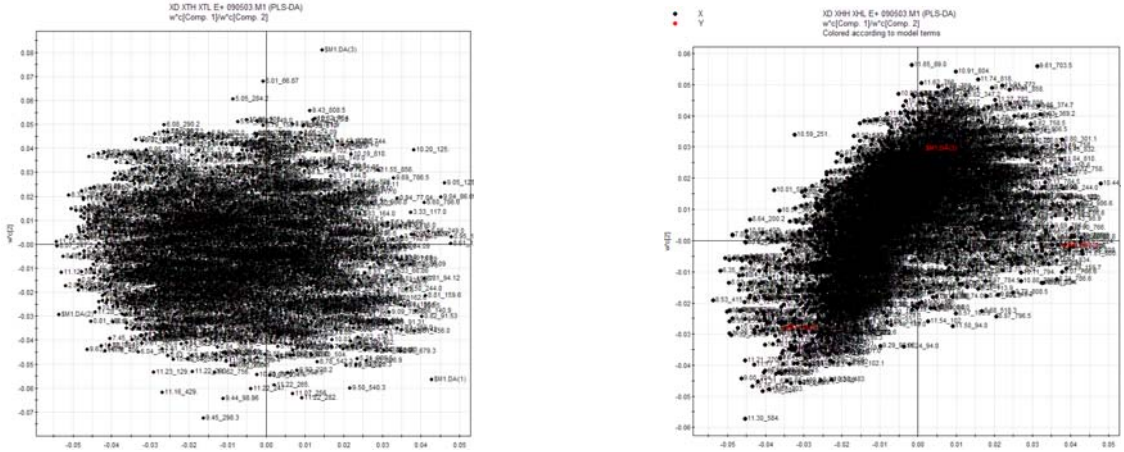

Fig.4 Loading plot of PLS-DA performance for biomarker recognition, which helped to screen out potential biomarkers caused by the dose of TBBPA or HBCD. (Left: loading plot of control group and TBBPA dosed group, ESI+; Right: loading plot of control group and HBCD dosed group, ESI+)

Fig 5. Level of the component m/z 415.3532 at 8.53 min in control group and HBCD doused group, a distinct increase tendency was found after HBCD exposure.

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