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Full Length Research Paper

Study on metabolism of N-Butyl Benzyl Phthalate (BBP) and Dibutyl Phthalate (DBP) in *Ctenopharyngodon idellus* by GC and LC-MS/MS

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To deeply study the toxic effect and mechanism of Dibutyl Phthalate (DBP) and Butyl Benzyl Phthalate (BBP) in fish, their *in vivo* and *in vitro* metabolism was investigated in this study. At first, a gas chromatography method was developed for simultaneous determination of DBP and BBP in blood serum, hepato-pancreas homogenate, intestine homogenate and hepatocyte medium. Then the main metabolites of the two PAEs chemicals were identified in *Ctenopharyngodon idellus* tissues by HPLC-MS/MS. And the results showed that DBP and BBP were metabolized quickly in blood serum, intestinal tract and hepato-pancreas homogenate, especially in the serum and the main metabolites were monobutyl phthalate (mBP) and monobenzyl phthalate (mBzP). However, they were not metabolized significantly in hepatocyte medium and the main metabolites in urine were the corresponding mono-phthalates and their conjugates of glucuronic acid. These studies indicated that DBP and BBP were hydrolyzed into mono-esters in some tissues in varying degrees, which were metabolized into corresponding conjugates combined with glucuronic acid later, and that will contribute to the elimination of PAEs from *C. idellus*. The blood and intestine were the first metabolized location of PAEs chemicals absorbed through fish's gill and intestine.

Key words: *Ctenopharyngodon idellus*, PAEs, GC, LC-MS/MS.

INTRODUCTION

As important classes of organic compounds, Phthalate esters (PAEs) were widely used as plasticizers in a variety of consumer products and household industries (Peakall, 1975). But phthalate esters are not chemically bound to the plastics, so they can be released easily from products and migrate into the aquaculture water that comes into direct contact. And it was reported that aquaculture water contaminated with phthalates has been absorbed by fish and represent toxicity to fishes (Li et al., 2008). Besides, some researches have found that PAEs exhibited estrogen-like activities (Borch et al., 2006; Pan et al., 2006), which have been classified into environmental endocrine disruptors. PAEs chemicals could influence the hormone metabolism in animals' reproduction and development process, thus, affecting the normal behavior of them (Makoto et al,. 2000; Pan et al., 2000; Salazar et al., 2004; Van Meeuwen et al., 2007). Therefore, the US Environmental Protection Agency (EPA) has classified six kinds of PAEs compounds, with DBP and BBP as priority control pollutants. Besides, three kinds of PAEs including DBP are also on China's major pollutants blacklist.

Until now, reported studies on the toxicity of PAEs were mainly focused on their environmental toxicology and aquatic toxicology (Sung et al., 2003; Chen et al., 2005; Pan et al., 2006). They have also represented immunity damages except for reproductive and developmental

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toxicity (Sung et al., 2003; Chen et al., 2005; Pei et al., 2009) and these chemicals could enter the human bodies through digestive system, respiratory system and skin contact. Besides, PAEs could affect people's offsprings through mothers' placenta and latex (Veronica et al., 2004). According to some literatures, the toxicity targets of PAEs chemicals were mainly focused on the animal's testis cells including Leydig cells, Sertoli cells and germ cells, which relate to the synthesis of steroid hormones and reproduction (Borch et al., 2006; Rao, 2008), while the toxic mechanism were mainly lied in the interference with aromatase activity and expression of the hormone transport-related genes and proteins (Andrade et al., 2006; Borch et al., 2006; Svechnikov et al., 2008) and these chemicals could also cause toxicity in algae, zebra fish and goldfish and result in prawn's blood cells apoptosis (Sung et al., 2003; Chen et al., 2005; Chen et al., 2008; Hu et al., 2008; Lin et al., 2008). Therefore, it is necessary to investigate metabolic ways in fishes to assess the environmental and aquatic risk from the potential exposures to phthalates and its metabolites.

Recently, some reports have clarified the PAEs metabolites toxicity on human beings (Makoto et al., 2001, Wang et al., 2006, Wang et al., 2007). And there were also some reports about the metabolism of PAEs in animals and micro-organisms (Li et al., 2005, Li et al., 2007, Wu et al., 2009). However, few articles were published on the metabolism in freshwater fish except for a study on *in vivo* pharmacokinetics and metabolism of analogs of PAEs in *coho salmon* (Barron et al., 1990). Therefore, for further investigation of the toxic effects of PAEs and its metabolites to freshwater fish, our studies have revealed *in vivo* and *in vitro* metabolic characteristics of DBP and BBP in *Ctenopharyngodon idellus,* which would provide references for PAEs safety evaluation and eco-toxicology research.

MATERIALS AND METHODS

The *C. idellus*, with the weight range from 450 to 550g, were obtained from YUETENG pisciculture (Hangzhou, China) and kept in the glass tank with recycling freshwater before experiment and the fishes were fed with a commercial feed ad libitum once a day. The water temperature was set as 25 ± 2℃ and fishes were maintained with a photoperiod of 12 h light, 12 h dark and the *C. idellus* hepatocyte line (No. BYK-C12-01), acquired from Fisheries Animal and Plant Pathogen Database (Shanghai Ocean University), Ministry of Agriculture, and was cultured in M199 medium (28℃ and 2% CO_2), which was provided by the Lan Bao Biological experiments Products Co., Ltd. (Hangzhou, China).

DBP and BBP, including their metabolites (mBP and mBzP) were all purchased from Shanghai J and K Chemical Co., Ltd. (purity＞99%). The internal standard was benzyl benzoate (obtained from SIGMA). Methanol, ethyl acetate and n-hexane were HPLC grade, which were obtained from Milk Chemical Instrument Co., Ltd. (Hangzhou, China).

Analytical instruments and methods

The gas chromatography assay used for determination of PAEs chemicals in the tissues was done by modification of the related method (Li et al., 2008). The GC system was Agilent 6890 GC series equipment, with a Flame Ionization Detector and Capillary Column (HP-5, 30 m and 0.25 × 250 μm) and the carrier gas was high-purity helium, with a flow rate of 40 mL/min, while the combustion gases were high-purity hydrogen and air. In the study, the splitless injection model was used and the volume was 1 μL. The column temperature was maintained at 80℃ for 1 min at first, and then increased to 235℃ at a rate of 30℃/min and the temperature was maintained at 235℃ for 15 min and increased to 290℃ at a rate of 5℃/min. The temperatures of injection port and detector were 280 and 300℃, respectively.

LC-MS/MS system was carried out in the modified Li's method with an optimized mobile phase (Li, et al., 2007). The system consisted of Waters ACQuity UPLC, Thermal LCQ and ESI. The mobile phase was methanol - 0.2% Acetic acid, and the flow rate was 0.2 mL/min. The gradient Elution program was adopted in the research. During the first 8 min, the proportion of methanol was increased from 10 to 40%, and was later increased from 40 to 50% and 50 to 80% during 8 to 9 min and 9 to 15 min, respectively and was maintained at 80% during 15 to 19min. Finally, the proportion decreased from 80 to 10% during 19 to 20 min. And UV detection wavelength was set at 254 nm. The column was ACQuity UPLC BEH-C_{18}（2.1 × 50 mm, 1.7 μm) and the temperature was maintained at 30℃. The flow rates of sheath gas and auxiliary gas were 10 and 2 L/min, respectively and the capillary column was 320℃.

Assay of DBP and BBP in tissue samples

In order to perform the test, four kinds of blank tissues (5 replicate samples for each tissue) were prepared, including serum, hepatopancreas homogenate, intestinal homogenate and hepatocyte medium. The DBP and BBP standard solution was added into 1 ml samples as mentioned earlier respectively, so as to make the final concentrations as 20 μg/mL. After that, all the samples were incubated at 30℃ for different time intervals. Then, internal standard and 3 ml ethyl acetate were added to each sample which was vortex-mixed and centrifugated (6000 rpm) for 5 min separately hereafter. The organic phase was transferred to a glass test-tube and the extraction repeated twice. After that, all the organic phase were collected together and evaporated to dryness at 45℃ under a weak stream of nitrogen gas. Then, the residue was reconstituted with 0.4 mL *n*-hexane, vortex-mixed for 1 min and centrifuged (18000 rpm) for 5 min. The 1 μL were injected directly into the GC system.

Gas chromatography analysis

DBP and BBP standard were dissolved in methanol, and were then diluted into a series of standard solutions at the concentration of 1.25, 2.5, 5.0, 10.0, 20.0 and 40.0 $\mu g/mL^{-1}$. Then the solutions aforementioned and internal standard (BB) was added into the blank serum, blank hepatopancreas homogenate, blank intestinal homogenate, and the suspension of hepatocyte. After which, the samples were processed according to the previous methods. The calibration curve was constructed based on peak-area ratios (y) vs. concentrations of spiked DBP and BBP (x), respectively. The standard curve equations and correlation coefficient (r) were calculated by the software Excel 2003 and the samples with concentrations of 2.5, 10.0, 40.0 $\mu g/mL^{-1}$ were selected as quality control (QC) and treated according to the methods earlier mentioned. Recovery of DBP and BBP with extraction method aforementioned was determined by comparing observed peak-area ratios in extracted serum and tissue samples with those of non-processed standard solutions. Precision of the method was

Table 1. Regression equation and correlation coefficients of DBP and BBP.

Variable	DBP		BBP	
Serum	$Y = 0.0547X + 0.059$	$r = 0.9996$	$Y = 0.0691X - 0.0025$	$r = 0.9997$
Hepato-pancreas	$Y = 0.0511X + 0.0432$	$r = 0.9996$	$Y = 0.0643X + 0.0065$	$r = 0.9999$
Intestine	$Y = 0.053X + 0.0462$	$r = 0.9991$	$Y = 0.0675X + 0.0505$	$r = 0.9993$
Hepatocyte	$Y = 0.0797X - 0.0307$	$r = 0.9999$	$Y = 0.0833X - 0.0171$	$r = 0.9999$

Table 2. Serum recovery and precision of DBP and BBP (n=5).

Chemical	Concentration (μg·ml^{-1})	Recovery (%)	Precision (%)	
			Intra day	Inter day
DBP	2.5	86.41±10.29	6.62	11.91
	10	91.76±8.15	5.88	8.88
	40	95.28±8.12	6.36	8.52
BBP	2.5	88.38±9.69	7.28	10.96
	10	90.13±8.51	5.81	9.44
	40	96.64±8.77	4.78	9.07

assessed by assaying five replicate QC samples aforementioned, respectively. Intra-day precision was evaluated at different times during the same day, while the inter-day precision was determined over five different days.

Mass spectra analysis and metabolite identifications

The DBP and BBP mixed standard solution were added to 1 ml serum, hepatopancreas homogenate, intestinal homogenate and the hepatocyte medium, respectively. Each sample was incubated at 30℃ for 120 min, and then 6 ml ethyl acetate was added to terminate the reaction. The organic layer was removed into a glass tube after extraction and evaporated to dryness in water bath with nitrogen gas and 1.0 ml methanol was added into the sample. After centrifugating for 3 min at 17000 r/min, 10 μl supernatant was injected into a LC-MS/MS.

Firstly, *C. idellus* were anesthetized by MS-222, and the catheter inserted and ligated into their ureter, after that, they were put into clean water. DBP and BBP were administered (0.5 g/kg body weight) by gavage when they woke up. Urine samples were thus collected for 12 h after administration and after acidification by dilute sulfuric acid, 1 ml urine was taken in centrifuge tube and 3 ml ethyl acetate was added into each sample. Then, the samples were vortex-mixed for 3 min and centrifugated for 5 min at 6000 r/min. The organic layer was transferred to the glass tube later. and the extraction repeated twice. Then, all the organic phase was collected together and evaporated to dryness at 45℃ under a weak stream of nitrogen gas. The residue was reconstituted with 1.0 mL methanol, vortex-mixed for 1min and centrifuged (18000 rpm) for 5 min. The 10 μL were injected directly into the LC-MS/MS system.

RESULTS

Validation of GC method

Evaluation of the assay was performed with calibration curves over the concentration range 1.25 to 40.0 μg DBP the calibration graph were calculated by weighted least squares linear regression and coefficients (r) and were calculated as followed. A good liner relationship ($r > 0.99$) between the peak-area ratios and the concentrations was observed (Table 1). The recoveries of DBP and BBP in the tissue were over 85%. The inter-day or intra-day coefficient of variation was less than 15% (n = 5). The recovery and precision of DBP and BBP in the tissue met the requirements of the detection (Table 2).

Tissue residues

The peak-area ratio of DBP and BBP and internal standard in tissue homogenates after metabolism with different intervals were substituted into the corresponding curve to calculate the concentration of the residue in tissue (Table 3). Although, DBP and BBP could not be metabolized significantly in the hepatocyte medium, they could be metabolized rapidly in the blood serum, hepato-pancreas and intestine homogenates. According to the results, the metabolic rates of DBP and BBP in blood serum were higher than in hepato-pancreas and intestine homogenates.

Metabolites identification

In order to identify the metabolites, DBP and BBP were analyzed by direct injection at first. The negative electrospray mass spectrum of them showed $[M-H]^-$ ions at m/z 277 and 312, respectively. Then the mixed standard solutions of DBP and BBP were added to 1 ml

Table 3. DBP and BBP concentrations of tissue within different intervals (μg·g^{-1}or mL^{-1}).

Variable	t /min	DBP	BBP
Serum	0	19.89±1.99	19.97±2.98
	5	6.56±0.75	6.78±0.59
	30	ND	ND
	120	ND	ND
Hepato-pancreas	0	19.81±1.28	19.36±2.35
	5	15.85±1.26	18.24±1.94
	30	6.58±0.51	10.23±1.12
	120	3.12±0.21	4.56±0.31
Intestine	0	20.15±1.61	19.58±1.74
	5	6.38±0.51	10.56±1.02
	30	3.23±0.27	6.14±0.53
	120	ND	2.21±0.18
hepatocyte	0	20.13±2.31	20.07±2.16
	5	19.12±1.81	20.05±1.86
	30	19.32±1.96	19.61±1.54
	120	18.98±1.84	19.69±1.94

ND: Not Detected.

serum, hepato-pancreas homogenate, intestinal homogenate and hepatocyte medium, respectively. DBP and BBP concentrations in the aforementioned tissues within different intervals (0, 5, 30 and 120 min) were determined. The detected results showed that DBP and BBP were metabolized quickly in blood serum, intestinal tract and hepato-pancreas homogenates, especially in the serum. However, they were not metabolized significantly in hepatocyte medium. By analyzing the mass spectra and mass-mass spectra of the metabolites in the earlier mentioned tissues, the structure of DBP and BBP metabolites were determined and their m/z were 221(M1) and 255 (M2), respectively. Compared with their parent compounds, the m/z of metabolites decreased at m/z 57, which indicated that the lose fragments of DBP and BBP were all butyl group. By analyzing the mass-mass spectra of M1 and M2, their m/z were 44 less than M1 and M2 (Parent compounds). Through comparing with their standards, M1 and M2 would be speculated as monobutyl phthalate (mBP) and monobenzyl phthalate (mBzP), which were hydrolyzed off a butyl group from their prototype compounds as Li et al. (2007) mentioned. The metabolites excreted in urines were characterized by HPLC-MS/MS. Four major metabolites (M3, M4, M5 and M6) of DBP and BBP were recovered in the urines. The mass spectra and mass-mass spectra of M3 and M4 were same as the metabolic characteristics of mBP and mBzP. So M3 and M4 were speculated as mBP and mBzP, respectively. Then m/z of M5 and M6 were 357 and 431 respectively, which were 176 (glucuronide) more than M3 and M4 according to mass-mass spectra. Therefore, M5 and M6 were inferred as glucuronic acid of monobutyl phthalate and monobenzyl phthalate, respectively (Figure 1).

These results showed that the DBP and BBP were not firstly metabolized by P450 oxidases in liver, but by some of the hydrolytic enzymes in the tissues. At the intestine, blood and other organs were important ways of exogenous compounds absorbed into the body by mouth or gill, biological transformation has occurred before they got into the liver. Therefore, it was concluded that DBP and BBP were mainly hydrolyzed and conjugated, and the blood and intestine were the first metabolized location of PAEs chemicals absorbed through the fish gill and intestines (Figure 2).

DISCUSSION

To date, most of reported investigations on PAEs were focused on the toxicity and detecting methods, including HPLC, GC, GC-MS and LC-MS (Lin et al., 2004; Jen et al., 2006; Li et al., 2008; Rios et al., 2010). Although, PAEs have been reported to have toxicity to reproductive and endocrine system of human beings and experimental animals, few studies were published on the toxicity of PAEs to fish. Sung et al. *(*2003) and Chen et al. *(*2005) reported the toxicity of several PAEs including DBP and BBP to the hemocytes and immune responses of giant freshwater prawn. In a laboratory experiment, three-spine stickleback *Gasterosteus aculeatus* were used to study the feeding behavior after exposure of butyl benzyl

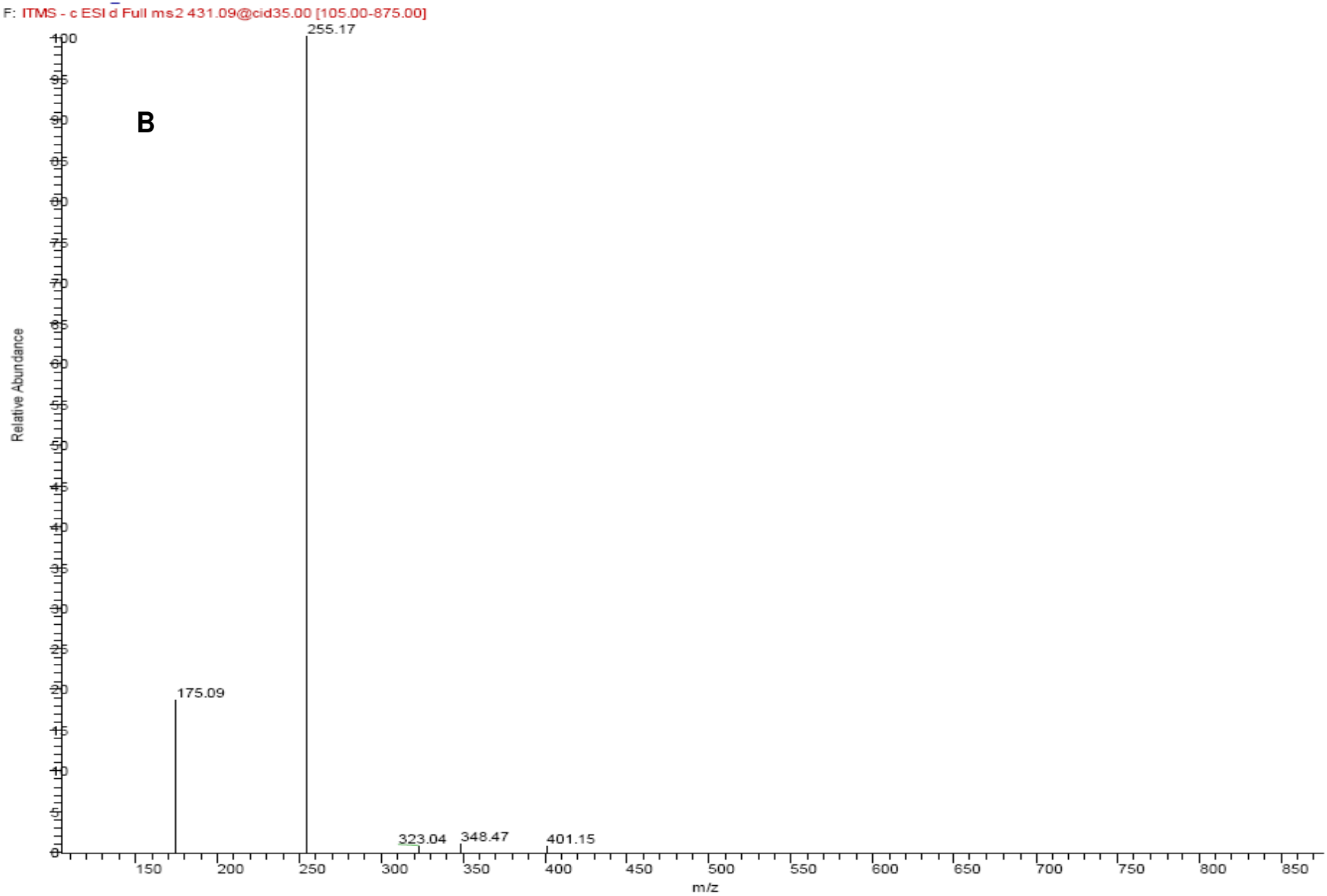

Figure 1. Mass spectra (A) and mass-mass spectra (B) of metabolites 6 (M6).

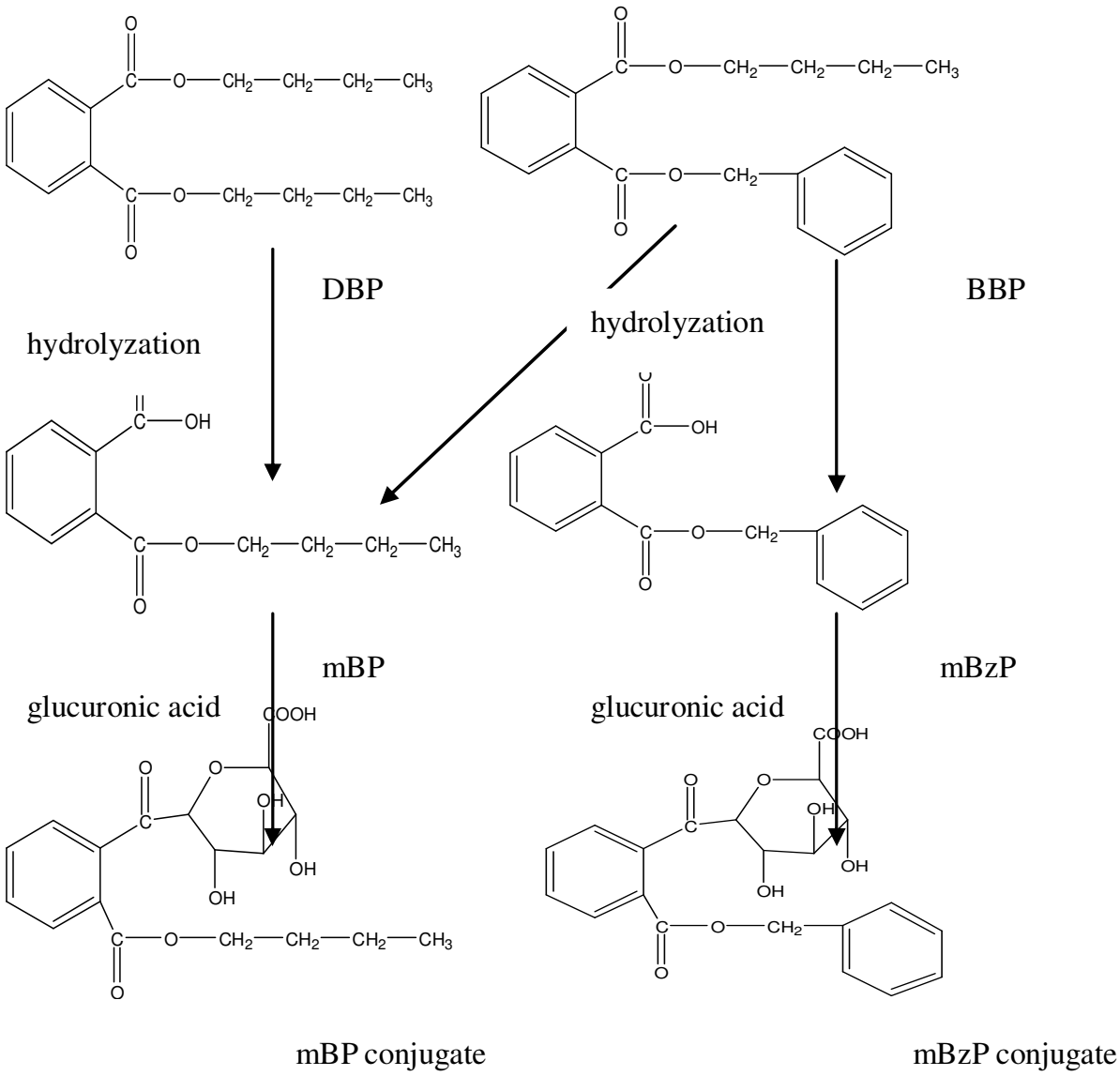

Figure 2. DBP and BBP metabolism in tissue and urine of *Ctenopharyngodon idellus*.

phthalate (BBP). Five weeks after exposure termination, the latency time to feeding differed from the control fish (Asa et al., 2004). Besides, BBP could also affect shoaling behavior and bottom-dwelling behavior in Three-spine Stickleback (Asa et al., 2002). Therefore, BBP may affect the fish's central nervous system according to the experiment as previously mentioned. As to DBP, physiological and biochemistrical characteristics of Brachydanio Rerio were also affected after exposure of DBP (Li et al., 2007).

In this study, GC method was used to detect DBP and BBP in several tissues of *C. idellus*. The detected results showed that DBP and BBP could be metabolized rapidly in the blood serum, intestine homogenate and hepato-pancreas. However, the two chemicals could not be metabolized significantly in the hepatocyte medium. Besides, DBP and BBP were metabolized faster in blood serum than in the intestine homogenates and hepato-pancreas. So, DBP and BBP would be metabolized easier by the enzymes of blood serum than intestine and hepato-pancreas. The results showed that the two chemicals could not be accumulated in *C. idellus*.

Through the usage of LC-MS/MS, the main metabolites we got included the corresponding mono-esters of DBP and BBP in blood serum, intestine homogenate and hepato-pancreas. However, the metabolites in *C. idellus* urine were detected and identified as corresponding mono-esters and conjugates combined with endogenous glucuronic acid and these conjugates have more soluble ability, which were more prone to excretion from *C. idellus* body. According to some reports, the metabolites of BBP had been studied by Nativelle et al. (1999) and Li

et al. (2007) in rat dosed with BBP. The metabolic characters of BBP in rat urine were similar to *C. idellus* in this study. However, the *in vivo* and *in vitro* metabolic pathways of DBP and DBP were not reported to date in freshwater fish, and as such were studied in our experiment. All of the results showed that DBP and BBP were firstly hydroxylated to corresponding mono-esters, which were conjugated with endogenous substances secondly. In any case, the metabolic mechanism and pathway of DBP and BBP in detail are still unclear and it would require further study.

In spite of the environmental concerns of PAEs, the investigations on their metabolism in fish were few. Some published results showed that the metabolites of DBP, mBP have stronger toxicity, which could lead to metabolic disorders of androgen synthesis in mouse testicular interstitial cells (Wang et al., 2006, 2007) and result in endocrine disorders. So, the toxicity of PAEs could be enhanced when they were metabolized by aquatic organism in water environment. Meanwhile, some researches indicated that PAEs were easy to be metabolized by microorganism in the environment (Li et al., 2005; Wu et al., 2009). Because of the stronger toxicity of these metabolites, they would raise the risk to the ecological system. Furthermore, when the PAEs metabolites was transferred through the food chain and entered human beings from the environment, all these chemicals would have a great impact on the reproductive and endocrine system of humans. Therefore, the metabolic kinetics and residue law of PAEs metabolites in the environment and fishes requires more research. The migration law and complex toxicity of PAEs metabolites should also be further investigated.

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