www.trjfas.org
ISSN 1303-2712
Turkish Journal of Fisheries and Aquatic Sciences 11: 253-259 (2011) DOI: 10.4194/trjfas.2011.0210

Comparative Studies on Toxicokinetics and Residues of Dimethyl Phthalate (DMP) in Tilapia（*Oreochromis niloticus* × *O. aureus*）at Different Water Temperatures

Jian Ge[1,*], Mingkui Li[1], Fang Lin[1], Jin Zhao[1], Xianjun Dai[1]

[1] China JiLiang University, College of Life Sciences, Hangzhou, Zhejiang, 310018, China.

* Corresponding Author: Tel.: +86.571 86914449; Fax: +86.571 86914449;
E-mail: gejian16888@163.com

Received 14 June 2010
Accepted 23 December 2010

Abstract

The study was conducted to evaluate the toxicokinetic disposition and residue of Dimethyl phthalate (DMP) in tilapia at different water temperatures (18ºC and 28ºC) by RP-HPLC method. After oral administration of DMP with a single does of 20mg/kg body weight, the tilapia's plasma, muscle, brain, skin and myocardium were sampled separately at different time to determine the concentration of DMP. Then the data were analyzed using 3p97 Pharmacokinetics software written by the Chinese Society of Mathematical Pharmacology, which indicated that the absorption, distribution and elimination half-life at 28ºC were 0.020 h, 0.19 h and 40.16 h, while the absorption half-life and elimination half-time were 3.01 h and 57.62 h at 18ºC. That's to say, the DMP absorption rate was faster in tilapia at 28ºC than that at 18ºC. And the serum concentration-time curve (AUC) was 30.13μg h ml^{-1} at 28ºC, while the T-peak was 0.074 hrs and the C-peak were 2.06μg·ml^{-1}; whereas, the corresponding situations at 18ºC were 74.77 μg·h·ml^{-1}, 13.51 hrs and 0.76μg·ml^{-1}, respectively. No matter how, the DMP residue was detected after oral administration for 360 hrs at both water temperatures, but the research had revealed that water temperature had great impact on the DMP absorption, distribution and elimination in tilapia.

Keywords: Tilapia（*Oreochromis niloticus*×*O.aureus*, Dimethyl phthalate (DMP), RP-HPLC, toxicokinetics, residue.

Farklı Su Sıcaklıgı Altında Tilapia （*Oreochromis niloticus* × *O. aureus*）Vücudundaki DMP Farmakokinetiği ve Kalıntıları Karşılaştırılma Analizi

Özet

Bu çalışmada RP-HPLC yöntemi kullanarak farklı sıcaklık altında (18ºC and 28ºC) tilapia vücudundaki Dimetilfitalat (DMF) kalıntıları ve farmakokinetiği ölçülmüştür. Tilapiyaya oral yolla 20 mg/kg DMP verilmesinden sonra, farklı zaman noktalarındaki plazma, kas, beyin, cilt, kalp'in DMP konsantrasyonu örneği alınıp analiz edilmiştir. Sonuçlara 3p97 farmakokinetik yazılım kullanılarak deneysel verileri analiz edilmiştir. DMP alımı sonrasında Tilapiyalarda yüksek sıcaklık altındaki vücut konsantrasyonu, düşük sıcaklıktan daha yüksek olduğunu göstermektedir. Emilim oranı hızlı emme yarı ömrü 0.020h.dagılım yarı ömrü 0.19 h, eliminasyon yarı ömrü 40.16 h, düşük sıcaklık altındakı emme yarı ömrü ve eliminasyon yarı ömrü ayrı ayrı 3.01 h ve 57.62 h.yüksek sıcaklık altında plazma konsantrasyonu-zaman eğrisi AUC alanı 30.13 μg h ml^{-1}.maksimum zamani 0.074 h. maksimum konsantrasyonu 2.06 μg ml^{-1};düşük sıcaklık altında ayrı ayrı 74.77 μg h ml^{-1}, 13.51 h ve 0.76 μg ml^{-1} aynı zamanda farklı su sıcaklıgı altında yedirdikten 360h sonra kas cilt beyin ve kalpdan DMP kalıntıları bulunuyor.ama farklı su sıcaklıgı altındakı Farmakokinetik ve kalıntılar Karşılaştırılma analizi su sıcaklıgının DMP'in tilapia vücudundaki emme,dağılım ve eliminasyona önemli etkisi olduğunu gösteriyor.

Anahtar Kelimeler: tilapia, DMP, HPLC, farmakokinetik, kalıntı.

Introduction

As important plastic-modifying additives, Phthalic Acid Esters (PAEs) are widely used in the production of plastic plasticizer, agricultural chemicals, coating material, dyeing and finishing, as well as cosmetics and spices. And along with the worldwide consumption of these products by human beings, PAEs have become largely diffused in the environment (Wu *et al*., 1999; Lin *et al.*, 2004; Chen *et al.*, 2007; Yang *et al.*, 2008). But as a kind of endocrine-disrupting compounds, PAEs possess estrogenic activities, which can influence the hormone metabolism in animals' reproduction and development process, thus affect the normal behavior of these animals. Further more, the bioaccumulation

of PAEs in organisms will bring potential threats to aquatic and human beings through food chain transfer (Van Meeuwen *et al*., 2007; Salazar *et al*., 2004; Foster *et al*., 2000; Ema *et al*., 2000). Therefore, the US EPA have classified six kinds of PAEs compounds, including DMP as priority control pollutants (Wang, 1995), and three kinds of PAEs, including DMP are also on China's major pollutants blacklist (Hu *et al*., 2003).

The tilapia (*Oreochromis niloticus* × *O. aureus*) is one of the commonly cultured fishes worldwide, which is well-known for its rapidity in breeding, convenience in farming and strong disease resistance, as well as its easy food processing and comparatively environment-friendly. China is the biggest aquiculture country in the world, which is also the major tilapia-producing country. In 2000, the production capacity of mainland China has already achieved 629,000 tons, and the total output of tilapia worldwide may achieve 2 million tons in 2010.

In this research, the toxicokinetic disposition and residue of DMP in tilapia have been well observed, so does the influence of the water temperature. This study was designed to reveal the disposing process and predict the toxicity mechanism of DMP in the fish. So far, the study has not been reported before, therefore the study will provide theory basis for aquatic toxicology, fishing sources protection and aquatic products safety.

Materials and Methods

Chemicals and Preparation

The standard Dimethyl phthalate (DMP) (purity ≥99%) was purchased from J&K Chemicals Ltd, Shanghai, China. All chemical agents, including methanol and acetonitrile for the analysis of DMP were of the HPLC grade, which were all obtained from Hangzhou Milk Chemical Instrument Co., Ltd, China. DMP was dissolved in corn oil to a concentration of 20 mg/ml as stock solution, which was stored at room temperature prior to the experiment.

Animals

Two hundred disease-free tilapias (mean body weight, 360±20 g) were obtained from YUETENG pisciculture (Hangzhou, China) and kept in flow through glass tank and acclimatized for one week before the experiment. Then these tilapias were divided into two large groups with 100 individuals for each according to the water temperature: 18°C and 28. In each large group, tilapias were further divided into two small subgroups: one for toxicokinetics and residue research, the other group for blank control. The water quality in tanks was tested daily. The pH was approximately 7 and the oxygen level was more than 10 mg/L due to the inflation pump. The water temperature was controlled at 18±1°C and 28±1°C with heating bars in different seasons. All the fish were supplied with a DMP-free commercial diet before administration. On the day before study, the fish were not fed.

RP-HPLC Method

The HPLC system used in this research was SHIMADZU-20AT series equipment, with a UV detector and a Zhida N2000 instrument workstation written by Zhejiang University. DMP separation was achieved on a SHIMADZU ODS C_{18} reversed-phase column (5 μm, 150 mm×4.6 mm) which had an ODS C_{18} pre-column (5μm, 12.5 mm×4.6 mm). The mobile phase was composed of acetonitrile - water (40:60, v/v) at a flow-rate of 1 ml/min. The column temperature was kept at 30°C and the detector wavelength was set at 235 nm.

Assay of DMP in Plasma and Tissues

A plasma sample (0.6 ml) was transferred to a 10 ml tapered glass tube with a screw cap and vortex-mixed for 1 min, then 3 ml ethyl acetate was added. The tube was vortex-mixed for 5 mins. After centrifugation (4024 g) for 5 mins, the organic phase was transferred to a glass test-tube. And this extraction was repeated twice. All the organic phase were combined and evaporated to dryness at 45°C under a weak stream of nitrogen gas. The residue was reconstituted with 0.1 ml mobile phase (acetonitrile-water), vortex-mixed for 1min and centrifuged (3,6220 g) for 5 min. The 20 μl were injected directly into the RP-HPLC system.

The analysis of tissue samples (muscle, skin, brain and myocardium) was carried out based on the method of plasma analysis, with some modification to suit different tissues. Some of the tissue samples (0.6 g homogenates of muscle and skin; 0.2 g homogenates of brain and myocardium) were transferred to a 10 ml tapered glass-tube. The following steps were similar to the plasma analysis procedure, except that the extraction of tissues for analysis was pretreated with solid-phase extraction column (SPE), which was made of 1 g diatomite with 1cm anhydrous sodium sulfate on its surface and cleaned with 10 ml *n*-hexane before elution. After dryed under a weak stream of nitrogen gas, the residues were reconstituted with 0.2 ml *n*-hexane and vortex-mixed for 1min. The *n*-hexane resolution was added to SPE cartridge and eluted with 8 ml mobile phase (1:4 ethyl acetate and *n*-hexane, v/v). The elution was collected in glass-tube and evaporated to dryness at 45°C under a weak stream of nitrogen gas. Then residue was reconstituted with 0.1 ml mobile phase (acetonitrile-water), vortex-mixed for 1 min and centrifuged (3,6220 g) for 5 min. The 20 μl were injected directly into the RP-HPLC system.

Calibration Curve

DMP standard solutions were prepared with methanol. Six concentrations of DMP for the calibration curve were prepared by dilution from the stock solution. The serial solution (0.6 ml each concentration) were evaporated to dryness at 45°C under a weak stream of nitrogen gas. Then 0.6 ml of DMP-free plasma and homogenate (muscle and skin) were added. The calibration range was 0.05-10 μg DMP per milliliter. Calibration standards were extracted and assayed according to the method mentioned above. The calibration curve was constructed based on peak-area of DMP (*y*) *vs* concentrations of spiked DMP (*x*). Then the standard curve equations and correlation coefficient (*r*) were calculated by the software Excel 2003.

0.2 ml serial solution (each concentration) were evaporated to dryness at 45°C under a weak stream of nitrogen gas. Then 0.2 ml of DMP-free brain and myocardium homogenate were added. And the other steps were identical to the plasma process.

Extraction Recovery and Precision

Blank plasma and tissue homogenate were spiked with the standards of DMP at low (0.05 μg/mL), medium (0.5 μg/ml) and high (5.0μg/mL) concentrations, which were used as quality control samples (QC) later within the calibration curve. Recovery of DMP with extraction method above was determined by comparing observed peak-area ratios in extracted plasma and tissue samples to those of non-processed standard solutions.

Precision of the method were assessed by assaying five replicate QC samples above, respectively. Intra-day precision was evaluated at different times during the same day. Inter-day precision was determined over five different days.

Toxicokinetics and Residue Analysis

In the experiment, fish were given a single dose of 20 mg/kg body weight DMP. After administration, all the fishes were fed ad libitum a commercial feed without DMP. The control groups were fed with DMP-free commercial feed. After oral administration, five fish were sampled per each time point. 2 ml of blood was randomly taken from the caudal vein at 0.083 h, 0.17 h, 1 h, 2 h, 8 h, 24 h, 72 h, 144 h after oral gavages, using evacuated syringes containing heparin to prevent clotting. Then the plasma was stored at -20°C after sampling until assayed. And the fishes were again used for tissues sampling at the time point 1 h, 2 h, 8 h, 24 h and 72 h after taking blood. Meanwhile, another 2 time points were added for tissues sampling, which were at 120 h and 360 h with the oral administered fish. All the samples were treated by the same method described above. The concentration of DMP in each plasma and tissue sample was calculated using the calibration curve. The parameters of toxicokinetics and residue were calculated using the 3P97 computer program written by the Chinese Society of Mathematical Pharmacology and Excel 2003 software.

Results

Specificity of Chromatograms

The RP-HPLC chromatogram peaks of DMP assayed by the standard solution was 7.432 min (Figure 1A). No significant interfering peak appeared for normal plasma and tissue samples (Figure 1B). Blank plasma and tissue samples spiked with DMP also yielded chromatogram peaks identical to that by the standard solution. A chromatogram of a plasma and tissue sample obtained after oral DMP administration showed that identical DMP peak was clear and strong without any endogenous substances at the corresponding time (Figure 1C and 1D).

Calibration and Validation of the Method

Evaluation of the method was performed with a calibration curve over the concentration range 0.05-10.0 μg ml^{-1} (g^{-1}) DMP. The slope and intercept of the calibration graph were calculated by weighted least squares linear regression. The regression equation of the curve and correlation coefficients (*r*) were calculated as followed. A linear relationship between peak-area and concentrations was good (Table 1).

The lower limit of quantification (LLOQ) was found to be 0.05 μg ml^{-1} (g^{-1}) for DMP in plasma and tissues. The intra-day and inter-day precisions were less than 10.0% for all three concentrations. Extraction recovery of DMP at concentrations of 0.05, 0.5 and 5.0 μg ml^{-1} (g^{-1}) in plasma and tissues were more than 85.0%, respectively. All the datum of DMP proved good precision and extraction recovery of the RP-HPLC method developed (Table 2).

Toxicokinetics and Residues at Two Water Temperatures

After oral administration of DMP at 20 mg/kg dose, DMP were determined at a series of time points within 360hrs in plasma and tissues under each temperature (18°C and 28°C). Plasma-concentration vs. time curves detected in the tilapia at the two temperatures were given in Figure 2. The plasma DMP concentration-time curve was fitted to a two-compartment open model at 28°C, whereas the curve was fitted to a one-compartment open model at 18°C. According to these concentration-time data and curves, the parameters of toxicokinetics were calculated by the computer software 3p97. And all the parameters for DMP at two temperatures were presented in Table 3. The absorption half-life ($t_{1/2Ka}$) of DMP was 3.01 h and 0.020 h at 18°C and 28°C,

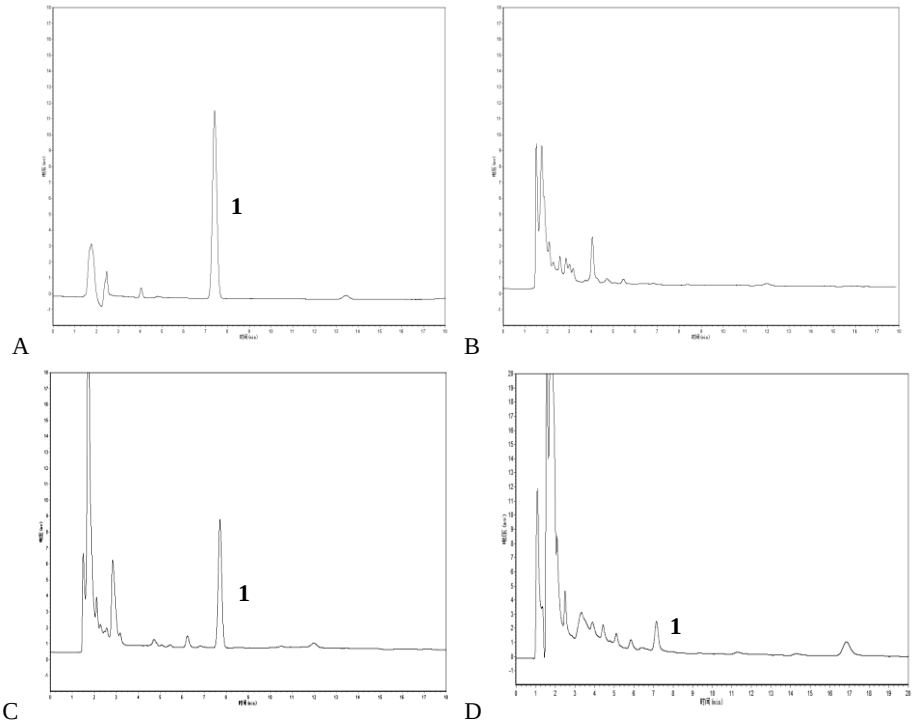

Figure 1. DMP standard solution chromatogram (A); Blank plasma chromatogram (B); Muscle chromatogram after the oral administration of DMP (C), Brain chromatogram after the oral administration of DMP (D)
Peak 1: DMP

Table 1. Tilapia in different tissues DMP curve equation and correlation coefficient

Organizational types	Curve equation	Correlation
Plasma	Y =111522X – 10387	r =0.9993
Muscle	Y =119301X – 3312.8	r =0.9999
Skin	Y = 116261X + 2623	r =0.9998
Brain	Y = 36113X + 1406.4	r =0.9998
Myocardium	Y = 39767X – 1104.3	r =0.9999

Table 2. Plasma and muscle recovery and precision of DMP (n=5)

Sample	Concentration $\mu g \cdot ml^{-1}$ (g^{-1})	Recovery (%)	Precision(%)	
			Intra day	Inter day
Plasma	0.05	88.41±8.59	7.82	9.72
	0.5	92.86±8.05	5.98	8.67
	5.0	95.36±7.27	7.62	8.86
Muscle	0.05	86.42±8.58	7.88	9.93
	0.5	90.31±8.15	5.28	9.04
	5.0	95.36±8.27	4.28	8.67
Skin	0.05	85.51±7.13	7.54	8.34
	0.5	89.87±8.61	6.46	9.58
	5.0	90.23±5.92	4.35	6.56
Brain	0.05	88.61±8.13	8.38	9.18
	0.5	91.87±8.51	6.26	9.26
	5.0	90.33±5.82	4.25	6.44
Myocardium	0.05	86.51±7.02	6.54	8.11
	0.5	88.77±7.61	5.36	8.57
	5.0	90.56±6.92	4.28	7.64

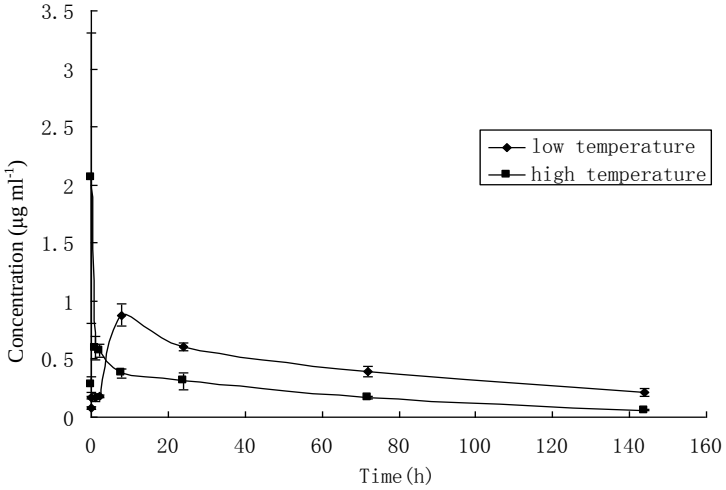

Figure 2. Different water temperature plasma DMP concentration - time curves.

Table 3. Metabolic kinetics of DMP in tilapia at different water temperatures

Parameters	Units	18ºC	28ºC
Absorption half-life $T_{1/2Ka}$	h	3.01	0.020
Distribution half-life $T_{1/2\alpha}$	h	/	0.19
Elimination half-life T1/2β	h	57.62	40.16
Concentration - time curve area AUC	$\mu g \cdot h \cdot ml^{-1}$	74.77	30.13
Peak time T_p	h	13.51	0.074
Peak concentration C_{max}	$\mu g \cdot ml^{-1}$	0.76	2.06

respectively. The elimination half-life ($t_{1/2\beta}$) of the chemical was 57.62 h at 18°C and 40.16 h at 28°C. The time of reaching maximum plasma concentration (Tp) and the maximum plasma concentration of DMP were also affected evidently by increased water temperature. Tp was 13.51 h at 18°C and 0.074 h at 28°C; Cmax was 0.76 and 2.06 μg ml^{-1} at 18°C and 28°C, respectively. The AUC was 74.77 $\mu g \cdot h \cdot ml^{-1}$ at the lower temperature and 30.13 $\mu g \cdot h \cdot mL^{-1}$ at the higher temperature. These results showed that the effect of water temperature on the toxicokinetics of DMP in tilapia was significant.

At two water temperatures, the residue of DMP in tissues is still above the detection limit within 360 h, and the concentration of DMP were both higher in the four tissues than in plasma at corresponding time. And the DMP concentration in tissues vs. time and the elimination half-lives ($t_{1/2\beta}$) are listed in Table 4 and Table 5. The concentrations of DMP in the tissues were higher at 28°C than at 18°C during 72 h. At the lower temperature, the $t_{1/2\beta}$ was longer than that in the higher temperature group. In lower temperature groups, DMP level was found to be higher in myocardium than that in the other tissues. And DMP concentration in myocardium was detected to be the highest at 360h after oral administration at 28°C.

Discussions

Choice of Analysis Method

Nowadays, there are many studies on the detecting methods for DMP, such as adoption of GC and GC-MS methods to measure the PAEs in fish's muscle (Tan and Lin, 2007; Li *et al.*, 2008). However, GC and GC-MS instruments are more expensive and less popular than HPLC. But reports on usage of RP-HPLC to detect the DMP in biological tissues are rare. Meanwhile, the toxicokinetic disposition of DMP in fish have never been reported before. In this study, the DMP concentration in tilapia's plasma, muscle, brain, skin and myocardium was determined using HPLC, and the toxicokinetics of DMP in tilapias was thus established according to these datum, so as to provide theory basis for routine detection of DMP and food safety.

At the beginning, several mobile phases were selected to separate DMP and endogenous co-eluents in plasma and tissues, including acetonitrile-water (50:50, v/v) and acetonitrile-water (30:70, v/v). However, only when the mobile phase was acetonitrile-water (40:60, v/v), DMP in plasma and tissues could be well separated by the RP-HPLC. Furthermore, a number of extraction solvents were

Table 4. DMP residues in the tilapia tissues at 18ºC (n=5)

Time (h)	Muscle ($\mu g \cdot g^{-1}$)	Skin ($\mu g \cdot g^{-1}$)	Brain ($\mu g \cdot g^{-1}$)	Myocardium ($\mu g \cdot g^{-1}$)
1	0.96±0.14	0.69±0.084	1.93±0.96	1.78±0.77
2	0.86±0.11	0.58±0.14	1.24±0.84	1.75±0.74
8	0.32±0.048	0.54±0.32	0.84±0.47	1.72±1.10
24	0.31±0.052	0.49±0.022	0.28±0.021	0.51±0.35
72	0.23±0.057	0.38±0.051	0.25±0.071	0.47±0.29
120	0.19±0.068	0.35±0.096	0.25±0.10	0.42±0.092
360	0.14±0.030	0.071±0.010	0.19±0.078	0.33±0.18
$t_{1/2ke}$(h)	169.02	407.65	144.36	165.00

Table 5. DMP residues in the tilapia tissues at 28ºC (n=5)

Time (h)	Muscle ($\mu g \cdot g^{-1}$)	Skin ($\mu g \cdot g^{-1}$)	Brain ($\mu g \cdot g^{-1}$)	Myocardium ($\mu g \cdot g^{-1}$)
1	1.34±0.64	1.22±0.043	3.97±0.14	2.27±0.96
2	0.97±0.12	1.01±0.089	3.27±0.28	1.81±0.19
8	0.82±0.14	0.62±0.21	2.04±0.69	1.75±0.012
24	0.77±0.25	0.42±0.25	1.40±0.82	1.43±0.0018
72	0.42±0.10	0.28±0.021	0.96±0.07	0.86±0.033
120	0.34±0.10	0.11±0.015	0.39±0.051	0.61±0.15
360	0.11±0.088	0.16±0.027	0.21±0.088	0.16±0.010
$t_{1/2ke}$(h)	110.00	147.45	92.40	99.00

tried to extract DMP from plasma and tissue samples, including ethyl acetate, diethyl ether and chloroform. Ethyl acetate gave a high extraction recovery of DMP without any significant interference. And then two absorption wavelength of 220 and 235 nm in the UV spectrum were selected to detect DMP. However, interferences from endogenous substances were observed with detection at 220 nm. A detection wavelength of 235 nm proved to be the most suitable and was therefore selected for the assay.

Toxicokinetics and Residues

As DMP is classified as endocrine-disrupting compound, it will disrupt animals' endocrine function through hypothalamus-pituitary-gonadal axis, resulting in endocrine disturbance, and affect the normal reproduction and development of animals finally (Van Meeuwen *et al.,* 2007; Salazar *et al.,* 2004; Foster *et al.,* 2000; Ema *et al.,* 2000; Lin *et al.,* 2009). Besides, the animal's endocrine system is closely connected with immune system, so the DMP will also diminish the immunity because of the endocrine system disturbance, making animals liable to disease (Lin *et al.,* 2009). So the characteristics of toxicokinetics of DMP in animals do have great impacts on the toxicological effects of DMP.

Fish are cold-blooded animals, their physiological characteristics, such as blood circulation, metabolism and immunity system are deeply influenced by the water temperature (Hardie, *et al.,* 1994). That's why their physiological characteristics and resistance to disease varies a lot in different seasons. So the tests were performed at different water temperatures in the tilapia in this study, and from the results we can find that the DMP absorption and elimination in tilapias were slower at a lower temperature (18°C), so were the time and concentration to peak; but the DMP absorption, distribution and elimination were faster at a higher temperature (28°C), and so was the distribution to peripheral tissues after absorption, and the peak of plasma concentration was detected in the shortest time, which made it much easier for the DMP to penetrate into tilapia's brain, gonad and muscle, and that's how the toxicological effects worked. However, the elimination rate differed at different water temperatures. Though the absorption was faster at higher temperature, the elimination was faster, too. As it was a long-term process for the fish's absorption of the contaminations in aquatic environment, the toxicity maybe even worse at lower temperature than at higher temperature.

According to the comparison of the residues in tissues at different water temperatures, it's obvious to find that the water temperature is an important factor for DMP elimination in tilapias. During the early stage of the test for residues, though the DMP residues in fish were lower at a lower temperature, but after 360 h of elimination, the DMP residues decreased little at a lower temperature. This proves that the lower the water temperature is the slower the elimination rate will be, so the elimination of residues in different seasons should be different. In this study, all tissues tested are edible tissues, and the elimination rate differs in different seasons.

The tissue analysis shows that DMP was distributed extensively in the tilapia. It was obvious

that DMP distribution varied in different tissues at various water temperatures. And at both temperatures, more DMP was found in tilapia brain than in other tissues. This showed that DMP was prone to accumulate in brain and hurt the brain. At each temperature, the elimination half-life in skin was longer than in other organs. That indicated that tilapia skin may be regarded as the marker organ of DMP residue. On the other hand, the elimination half-life differed significantly between two temperature groups. In cold water, the value was higher than in warm water. So in cold water the longer half-life of DMP in tissues means a longer withdrawal period.

Acknowledgements

This work was supported by the Natural Science Foundation of Zhejiang Province (Y3080255), People's Republic of China.

References

Chen, Z.L., Huang, Y.K., Wang, G.M., et al. 2007. Detection method of phthalates of environment hormone in cosmetics. Environmental Science and Technology, 30(4): 43-47. doi: cnki:sun:fjks.0.2007-04-015.

Ema, M., Miyawaki, E. and Kawashima, K. 2000. Effects of dibutyl phthalate on reproductive function in pregnant and pseudopregnant rats. Reproductive Toxicology, 14(1): 13-19. doi: 10.1016/S0890-6238(99)00066-0

Wang, L.S. 1995. Chemistry of Environmental Pollution. Press of chemical industry, Beijing: 316-318.

Foster, P.M.D., Cattley, R.C. and Mylchreest, E. 2000. Effects of di-n-butyl phthalate (DBP) on male reproductive development in the rat: implications for human risk assessment. Food and Chemical Toxicology, 38(1): 97-99. doi:10.1016/S0278-6915 (99)00128-3.

Hardie, L.J., Fletcher, T.C. and Secombes, C.J. 1994. Effect of temperature on macrophage activation and the production of macrophage activating factor by Rainbow Trout (*Oncorhynchus mykiss*) Leucocytes. Developmental and Comparative Immunology, 18(1): 57-66. doi:10.1016/0145-305X(94)90252-6.

Hu, X.Y., Zhang, K.R. and Sun, J.H. 2003. Environmental contamination by phthalates in china. Chinese Journal of Health Laboratory Technology, 13(2): 9-14. doi: cnki:sun:zwjz.0.2003-01-001.

Li, X., Nie, X.P. and Pan, D.B. 2008. Analysis of PAEs in muscle tissue of freshwater fish from fishponds in Pear River Delta. Journal of Environment and Health, 25(3): 202-205. doi: cnki:sun:hjyj.0.2008-03-004.

Lin, X.T., Chen, M., Wang, X.Y., et al. 2004. Analysis of phthalate esters of environmental hormone in waste water treatment plant. Environmental Science and Technology, 27(6): 79-83. doi: cnki:sun:fjks.0.2004-06-031.

Lin, X.T., Wang, X.Y., Lu, W.T., et al. 2009. Research progress in metabolism of phthalate esters. Journal of Environment and Health, 26(2): 182-184. doi: cnki:sun:hjyj.0.2009-02-040

Salazar, V., Castillo, C., Ariznavarreta, C., et al. 2004. Effect of oral intake of dibutyl phthalate on reproductive parameters of Long Evans rats and pre-pubertal development of their offspring. Toxicology, 205(1): 131-137. doi:10.1016/j.tox.2004.06.045.

Tan, J. and Lin, Z.G. 2007. GC-EI-MS internal standard analysis of phthalic acid esters in fish. Acta Chimica Sinica, 65(24): 2875-2882. doi: cnki:sun:hxxb.0.2007-24-015.

Van Meeuwen, J.A., Weterburg, A.H. and Piersma, M. 2007. Mixture effects of estrogenic compounds on proliferation and pS2 expression of MCF-7 human breast cancer cells. Food and Chemical Toxicology, 45(11): 2319-2330. doi:10.1016/j.fct.2007.06.011.

Wu, P.G., Han, G.G., Wang, H.H. and Zhao, Y. 1999. An investigation on Phthalates in Drinking Water. Journal of Environment and Health, 16(6): 338-339. doi: cnki:sun:hjyj.0.1999-06-008

Yang, Q., Zhang, M.S. and Chen, W.S. 2008. Determination of 6 kinds of phthalate ester in edible oils by Gas Chromatography. Journal of the Chinese Cereals and Oils Association, 23(5): 171-174. doi: cnki:sun:zlyx.0.2008-05-039