Advance Journal of Food Science and Technology 7(5): 383-387, 2015
DOI:10.19026/ajfst.7.1328
ISSN: 2042-4868; e-ISSN: 2042-4876

Submitted: September 18, 2014 Accepted: October 01, 2014 Published: February 15, 2015

Research Article

Metabolism and Elimination of Oxytetracycline in Hepatopancreas of Chinese Mitten Crab Eriocheir Sinensis

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Abstract: The kinetic profiles of Oxytetracycline (OTC) in hepatopancreas of Eriocheir sinensis was examined in this research. The results showed that after a single-dose of 40 mg/kg body weight OTC at (18±1°C) by intramuscular administration, the hepatopancreas concentration-time curve of OTC could be best described by a one-compartment model with first-order absorption and a time lag: C = 69.45×(e-0.005(t-0.0387)-e-8.195 (t-0.0387)). The elimination half-life (t1/2β) was 130.71 h. It is suggested that a withdrawal time of 79.51d should be required before crabs are consumed safely.

Keywords: *Eriocheir sinensis*, metabolism, oxytetracycline, withdrawal time

INTRODUCTION

The Chinese mitten crab, *Eriocheir sinensis* (also known as river crab), is one of the most important aquatic products in China. With the enlargement of the cultivation scale, various diseases have emerged, which resulted in huge economic losses. Tremor Disease Agent (TDA) is one of the most hazardous pathogens. Based on our previous studies and molecular biology methods, TDA was thought to be a spiroplasm (Wang *et al*., 2003, 2004a). Oxytetracycline (OTC) was found to be very effective against TDA according to in vitro antimicrobial susceptibility test (Liang *et al*., 2009). OTC is the first fish antibiotic that approved by Food and Drug Administration (FDA) of USA, which has been widely used in control of aquatic animal diseases. Recently, much attention has been paid to safe medication in aquaculture, mainly focusing on fish and shrimp (Black *et al*., 1991; Chiayvareesajja *et al*., 2006; Poapolathep *et al*., 2008; Rigos *et al*., 2002). There is only a few work on pharmacokinetics of OTC in *E. sinensis*, which examined the drug concentrations in haemolymph and muscle (Feng *et al*., 2010, 2011). So far there is no report on OTC kinetics in hepatopancreas (the best edible parts of *Eriocheir sinensis*), which has the function of detoxification. In this study, the metabolism and elimination of OTC were investigated thoroughly.

MATERIALS AND METHODS

Animals: One hundred fifty healthy crabs (101.7± 6.1g) were purchased from Hongqi Farm of Taizhou in Jiangsu province. *E. sinensis* were kept in circular tanks (4.0×3.0×3.0 m) at 18±1°C for two weeks before the experiment.

Chemicals: Oxytetracycline hydrochloride of high purity (>99%) was obtained from Sigma-Aldrich Chemica Co. (St.Louis, MO, USA). Solvents (acetonitrile and methanol) were of HPLC grade (Tedia, Fairfield, OH, USA). Other chemicals including hydrochloric acid, phosphoric acid, perchloric acid, disodium hydrogen phosphate, triethylamine and EDTA were of analytical grade.

Drug delivery: Before giving the dose, crabs were surface disinfected with 75% alcohol. The drug was delivered through the arthrodial membrane in the hinge region between the fourth pereiopoda. The dosage was 40 mg/kg body weight, determined by former treatment experiment.

Sample preparation: Sampling time was as follows: prior to and after intramuscular administration of OTC at 5, 10, 15, 30 min, 1, 2, 4, 6, 8, 12, 24, 48, 72, 168 and 336 h, respectively. Ten crabs were collected and killed at each sampling interval. Hepatopancreas was dissected and kept frozen at -20°C until OTC analysis.

Samples were thawed at room temperature and broken up with electric homogenizer. 5.000 g of samples were weight accurately and put into a 50 mL centrifuge tube with 10 mL 1% perchlorate solution. The tube was vortexed for 15~30 s, then centrifuged for 10 min at 10000 rpm/min. The supernatant was transferred. The procedure was repeated for three more times with 15, 15, 10 mL perchloric acid solution to extract OTC completely. All the supernatants were

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collected and filtered (0.45 μm nylon membrane).The samples were purified by a solid phase extraction procedure performed with AccuBOND ODS-C_{18} (Agilent, 500 mg of 3 mL capacity). Columns were preconditioned with 5 mL of methanol, 2 mL of 5% EDTA and 5 mL ultrapure water. After this, columns were cleaned with 10 mL ultrapure and eluted with 20 mL 5% triethylamine methanol solution. The eluate was evaporated to dryness by rotary evaporator with water bath at 50°C, resuspended in 1 mL of mobile phase solvent. Before HPLC analysis, all the samples must be filtered (0.45 μm nylon membrane).

Chromatographic conditions: High performance liquid chromatography analysis was performed with an Agilent 1100 consisting of a quaternary pump (G1311A), an auto-injector (G1313A) and a variable wave-length detector (G1314A). Analytes (20 μL, sample volume) were separated on a ZORBAX SB-C18 250×4.6 mm i.d., 5 μm reverse phase silica column. The mobile phase was ACN-0.05 mol/L Na_2HPO_4 containing 0.001 mol/L EDTA (pH2.5) (18:82, V/V), degassed and filtered before use. The column temperature was maintained at 37°C, the flow velocity, 1.0 mL/min; the detection wavelength is 355 nm, the flow rate was 1 mL/min and the UV detector was used at the wavelength of 355 nm.

Data analysis: 3P97 (Chinese pharmacological society mathematics professional committee compiled) was used to analyze kinetic profiles of OTC according to the residual sum of squares and the minimum Akaike's information criterion value. The elimination characteristics of the drug were calculated by using the last four time points. Least-square regression analysis was applied to estimate the elimination slope (β). The elimination half-lives were obtained from the equation: $t_{1/2} = \ln 2/\beta$ (Baggot, 1977). Optimization of dosage regimen was calculated with the formula: $C_{min} = Dose \times e^{-\beta td}/V_{d(area)}$, where C_{min} is the minimum effective concentration and t_d is the duration of the effect (Doi *et al*., 1998).

Withdrawal period was determined as the time post-dosing that OTC level fell below the maximum residue limit (MRL, 0.1 μg/g) and calculated with withdrawal-time calculation program WT1.4, which was adopted by the Committee for Veterinary Medicinal Products (CVMP) (Blanchflower *et al*., 1997).

RESULTS STAND

HPLC assay: Under the conditions mentioned above, the retention time of OTC is about 5.344 min (Fig. 1 and 2). Though an impurity peak appeared in the sample, the target compound (OTC) was separated with good resolution (Fig. 2). Figure 3 showed that, the standard calibration curve for OTC was found to be

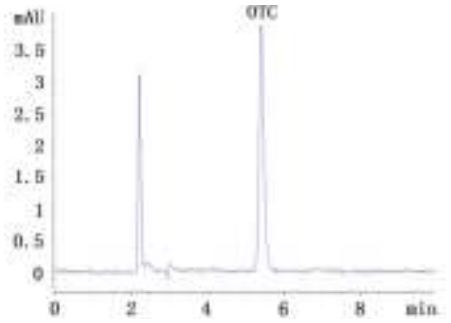

Fig. 1: Chromatograms of OTC standard (1μg/mL)

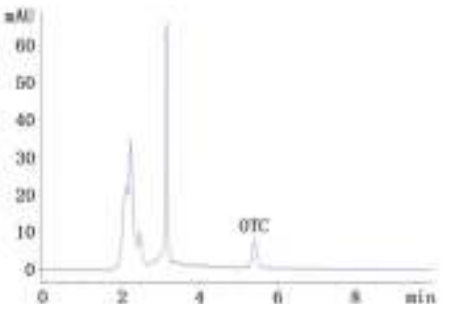

Fig. 2: Chromatograms of hepatopancreas sample (test sample)

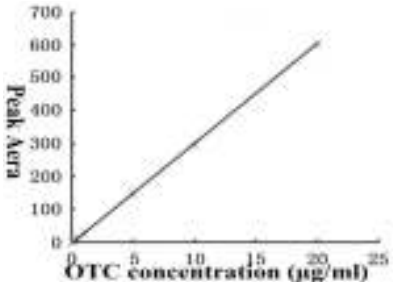

Fig. 3: Standard curve of OTC

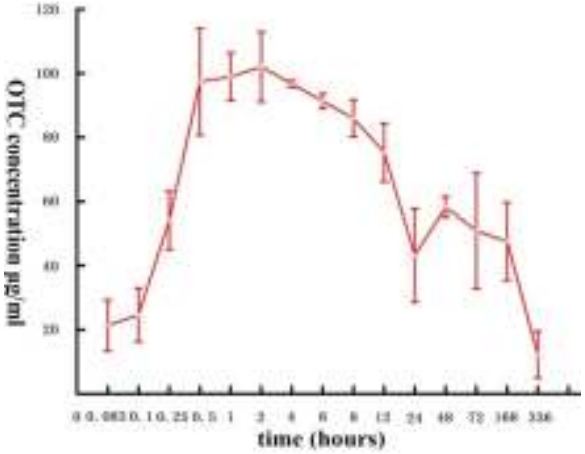

Fig. 4: OTC concentration-time profiles of hepatopancreas following a single intramuscular dose of 40 mg/kg body weight (18±1°C)

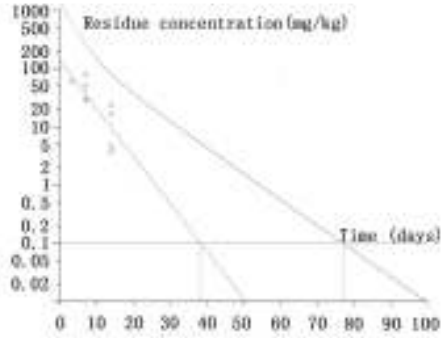

Fig. 5: Withdrawal time when the maximum residue limits was set at 0.1 mg/kg

Table 1: Pharmacokinetic parameters of OTC in hepatopancreas of *E. sinensis* after intramuscular injection (18±1°C)

Parameters	Unit	Value
Compartment model		
A	(μg/mL)	69.4500
K_e	(1/h)	0.00500
K_a	(1/h)	8.19500
T_0	(h)	0.03870
Non-compartment model		
$AUC_{0-\infty}$	(μg·h/mL)	16352.31
$MRT_{0-\infty}$	(h)	178.7900
CL_S	(L /kg /h)	0.002450
V_d/F	(L/kg)	0.460000

A: The zero time drug concentration intercepts of disposition and elimination; K_a, K_e: First order rate constants for drug absorption and elimination; T_0: time lag; $AUC_{0-\infty}$: Area under the concentration from zero to infinity; $MRT_{0-\infty}$: Mean residence time from zero to infinity; CL_S: The total body clearance; V_d/F: Apparent volume of distribution

linear over the range 0.05~20 μg/mL, with correlation coefficients >0.999.

Kinetic analysis: The hepatopancreas concentration-time curve was presented in Fig. 2. There were two peaks in the curve. OTC concentration reached the first peak (102.02±10.92 μg/mL) 2 h after dosing. In this stage, drug absorption is faster and then the concentration declined until 24 h. After that, the concentration started to rise again, the second peak (58.27±3.26 μg/mL) appeared at the 48 h, then the concentration dropped slowly over time (Fig. 4). The concentration-time curve was best described by one compartment open model with time delay. The theoretical equation is:

$$C = 69.45\times(e^{-0.005(t-0.0387)}-e^{-8.195(t-0.0387)}$$

The terminal elimination rate constant (β) is 0.005 h^{-1}. The corresponding elimination half-life ($t_{1/2\beta}$) is 130.71 h. The main kinetic parameters could be found in Table 1. The dosage was optimized based on those parameters, 41.85 mg/kg body weight was recommended.

Withdrawal time calculation: Referring to food safety standards of China, with the maximum residue limits (MRL, 0.1 mg/kg), the withdrawal time was calculated to be 79.51d (Fig. 5).

DISCUSSION

Perchloric acid was used as protein precipitant and the extractant for OTC from biosamples. Triethylamine was added to improve the chromatographic separation and avoid tailing phenomenon. EDTA functioned as chelator to remove any impure metal ions in chromatographic column, which could get better peak resolution (Oka *et al*., 2000).

The Limit of Detection (LOD) is 0.015 μg/mL, determined as 3 times of the baseline noise. The extraction recovery of OTC was 74.18 and 86.97% for samples spiked with 0.1 μg/mL and 0.2 μg/mL respectively. Coefficients of variation were 2.87 and 8.65% for intra-day and inter-day assays respectively (n = 6).

In former researches on liver tissue, two-compartment model was found to fit the experiment data (Wu *et al*., 2006; Zhang *et al*., 2008). While in this study, one-compartment model fitted better. Considering the differences of test conditions, sampling design, it is difficult to determine exactly which model is more appropriate. The non-compartmental model is not affected by the limitation of classical compartment model, which was applied for comparison between species.

The maximum OTC level was much higher than the values reported in *Scylla serrata* (32.12 μg/mL) (Chen *et al*., 2009) and *Paralichthyidae olivaceus* (13.78 μg/mL) (Ji *et al*., 2008). Tmax (2 h) was shorter than that observed in *Scylla serrate* (4 h) (Chen *et al*., 2009) and *Paralichthyidae olivaceus* (4 h) (Ji *et al*., 2008) respectively. The difference could be explained by factors such as medication administration route. In this study, the drug was delivered intramuscular injection. While oral administration was applied for the other two species, the first pass effect might play an important role in Tmax delay.

The Double Peak Phenomenon appearing in this study was also found in other researches, which suggested reabsorption of OTC in hepatopancreas (Guo *et al*., 2005; Li *et al*., 1997). The area under the concentration-time curve of terminal phase (the last four time points) contributed 67.61% to the area under the concentration from zero to infinity, which confirmed the existence of reabsorption in theory. The dose reabsorbed was unlikely to be from crustaceans exoskeleton, for OTC with strong capacity of chelating Ca^{2+}/Mg^{2+} couldn't release from exoskeleton within such a short period (2-14 d). It was also impossible to be explained by the slow release of OTC residues in the injection site, because of the open vascular system OTC would be absorbed completely soon after intramuscular injection. Warner (1977) considered that the main functions of the green gland (excretory organ) is not

expelling waste from body but keeping ion balance and reabsorbing selectively. Hence the phenomenon of two absorption peaks in the hepatopancreas may be linked with reabsorption mechanism of green gland.

The models of non-compartmental and compartmental were both applied for elimination rate constant calculation, which showed that the same result (0.005 h^{-1}) was achieved. It was verified that the one-compartmental model was best fitted. Compared with other fish species, OTC elimination rate in hepatopancreas of *E. sinensis* (18±1°C, 0.005 h^{-1} or 0.12 d^{-1}) was faster than that in liver of cold water species (Chinook salmon 15°C, 0.097 d^{-1} (Namdari *et al*., 1996); rainbow trout 10±0.5°C, 0.103 d^{-1} (Namdari *et al*., 1999); salmon 10±0.5°C, 0.068 d^{-1} (Namdari *et al*., 1999), but slower than that in liver of warm water species (grass carp 21±1°C, 0.34 d^{-1} (Zhang and Li, 2007). In crustaceans, the elimination half-life of OTC in *E. sinensis* was shorter than that in *Penaeus chinensis*（23-25°C, $t_{1/2\beta}$-16.12h) (Wang *et al*., 2004b) and in *Scylla serrata* (27±1°C, $t_{1/2\beta}$-71h) (Chen *et al*., 2009). It seems like that temperature might be the main factors influencing the elimination rate besides physiological structure differences between species.

One purpose of this study was to optimize dosage regimen. Our former research showed that the minimal inhibitory concentrations of OTC are 0.04 μg/mL. According with one compartmental model selected, t_d was calculated to be 719.38 h (nearly 30d).The optimized dosage is 41.85 mg/kg body weight, close to the dosage administrated (40 mg/kg body weight).

So far there are only two kinds of software's about withdrawal time calculation at home and abroad. The homemade one was only designed for two compartmental models with intravenous injection, which didn't apply to this study (Wang *et al*., 2009). The software WT-1.4 used here isn't subject to the limitations described above. 18±1°C was chosen in this experiment, for simulating the water temperature conditions in late spring and early summer during clinical therapy. Considering the effect of water temperature on OTC elimination, too long or too short period of withdrawal time will affect the farmers negatively. The withdrawal time was converted into units of degree-day value (°C-day) for ease of reference. Thus under the conditions of this study (water temperature 18±1°C, single dose of 40 mg/kg body weight by intramuscular injection), the withdrawal period of OTC in hepatopancreas of *E. sinensis* is 1432°C-day.

Future research will focus on population pharmacokinetics characteristics of OTC after different administration ways (oral administration and bath), taking various temperature conditions and different physiology situation (health and sickness) into account, which will provide theoretic support and practical reference to production.

ACKNOWLEDGMENT

This study was supported by grants from Colleges Science Key Research Project (ZD1109).

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