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ISSN 0792 - 156X

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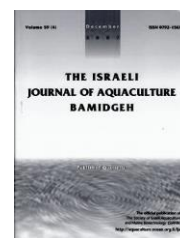


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## **Pharmacokinetics and Tissue Residence of Various Doses of Levamisole in Crucian Carp at Different Water Temperatures**

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**Keywords:** water temperature; dosage; levamisole; pharmacokinetics; residue

### **Abstract**

The pharmacokinetics of levamisole in crucian carp, including the absorption, distribution, and clearance rates in different tissues, were studied at different temperatures and doses for the theoretical basis of proper usage and withdrawal time. The fish were divided into three groups denoted A, B and C. The A and B groups were treated once at a dosage of 5 mg/kg levamisole, at  $19 \pm 1^\circ\text{C}$  and  $26 \pm 1^\circ\text{C}$  water temperature, respectively. The C group was treated at a dosage of 20 mg/kg levamisole at  $19 \pm 1^\circ\text{C}$  water temperature. Then plasma, muscle, hepatopancreas, and kidney tissues samples were collected and pretreated, then measured using an ultra performance liquid chromatography-ultraviolet method. Plasma data were analyzed using DAS 3.0 pharmacokinetics software. Total area under the curve ( $AUC_{0-t}$ ) and elimination half-life ( $t_{1/2z}$ ) had a negative correlation with water temperature, and a positive correlation with dosage. Apparent volume of distribution ( $V_z$ ) and total clearance rate ( $CL_z$ ) was positively correlated with temperature, and a negatively correlated with dosage. Levamisole was absorbed quickly to reach its peak concentration. The drug concentration and residence time in hepatopancreal and kidney were considerably higher and longer respectively than in the muscle and plasma suggesting that the hepatopancreas and kidney may be important metabolic organs for levamisole.

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#The first two authors contributed to the work equally.

## Introduction

As a common vermifuge of the imidazole and thiazole type (Cun et al., 2008), levamisole has been commonly used in animal husbandry for pigs, cows, and sheep because of its broad spectrum, high efficiency, low toxicity, and safety (Siegrid et al., 2003). The mechanism works to prevent reversion of fumaric acid to succinic acid by inhibiting the activity of the fumaric acid, which affects the process of the glyceic metabolism, to reduce the insect body ATP, which leads to body paralysis and death. Levamisole also plays a significant role in immunoregulation to strengthen immunity and increase animal growth. There have been many studies (Pereda et al., 2002; Cheng et al., 1991; Lin et al., 2003; Garcia et al., 1990; Galtier et al., 1983; Paulson & Feil, 1996; Xie et al., 1989; EI-Kholy & Kemppainen, 2005) on the pharmacokinetics of levamisole in terrestrial animals. It has been widely applied in the ornamental fish industry as an immunostimulant by improving phagocyte activity and neutrophilic granulocytes, stimulating the generation or secretion of lymphocytes, enhancing metabolic capability and lysozymes in the serum and inducing the generation of antibodies and complement proteins (Wang, 2018). Currently, more research reports concerning levamisole-induced immune enhancements and the bactericidal effects in fish have been published (Lipton et al., 2009; Siwicki, 1987; Xiang et al., 2002; Kajita et al., 1990; Huang et al., 2008; Wu et al., 1998) as well in sporozoa treatment (Chen, 2013), but less on levamisole pharmacokinetics in crucian carps. However, Levamisole hydrochloride is one of the effective drugs to treat myxosporiasis, and widely used in the prevention and cure of myxosporiasis in aquaculture industry (Liu & Wang, 2016; Wu et al. 2012 & Chen, 2013). The current study aims to investigate the pharmacokinetic parameters of levamisole in crucian carp and its absorption, distribution, clearance and residue (Syahidah et al., 2015,) at different water temperatures and various doses. A second aim is to explore the effects of water temperature and dosage on the metabolic residue of levamisole in crucian carp, thus providing a theoretical basis for dosage plans and the withdrawal time of levamisole.

## Materials and Methods

### *Fish.*

Healthy crucian carps *carassius auratus gibelio* 'CAS No.3', (weight  $200 \pm 15$  g) were purchased from Changchun Jiutai seed multiplication farm and used for our experiments. To meet the test conditions, the fish were divided into individual aquariums, and kept in at a water temperature of  $19 \pm 1^\circ\text{C}$  or  $26 \pm 1^\circ\text{C}$ . The fish were not fed during the experiment.

### *Solvent and solution preparation.*

Chromatographic pure ethyl acetate and methanol and analytic pure phosphoric acid, triethylamine, anhydrous sodium sulfate (dried for 4 hours at  $650^\circ\text{C}$ ), hydrochloric acid, ammonium hydroxide, and boric acid were used for preparing the solutions or sample treatments. Ultrapure water was used as test water. Levamisole hydrochloride with a purity of 99.8% was provided by the National Control Institute of Veterinary Bioproducts and Pharmaceuticals. Levamisole hydrochloride with a purity of not less than 98.0% was provided by Nanjing Nongmu Gaoke Biotechnology Co., Ltd.

Preparation of standard solutions: levamisole hydrochloride standard was dissolved in methanol to a constant volume of 100 mL to produce a standard levamisole stock solution of  $200 \mu\text{g/mL}$ . This was stored in a refrigerator, and away from light, at  $-18^\circ\text{C}$ . Before use, the stock solution was diluted in methanol to prepare  $0.1 \mu\text{g/mL}$ ,  $0.5 \mu\text{g/mL}$ ,  $2.0 \mu\text{g/mL}$ ,  $10 \mu\text{g/mL}$ ,  $50 \mu\text{g/mL}$ ,  $100 \mu\text{g/mL}$  and  $200 \mu\text{g/mL}$  standard working solutions. 6.18 g of boric acid was added as a buffer to 980 mL of water, then the pH was adjusted to a value of 9.0 with sodium hydroxide solution (10 M). Finally, water was added to reach a constant volume of 1000 mL.

### *Oral administration and sample collection.*

The fish were divided into three test groups: group A, maintained at water temperature of  $19 \pm 1^\circ\text{C}$  received one oral administration of levamisole at a rate of 5 mg/kg body weight; group B, maintained at water temperature of  $26 \pm 1^\circ\text{C}$ , received one oral administration of levamisole at 5 mg/kg body weight; group C, maintained at water temperature of  $19 \pm 1^\circ\text{C}$ , received one oral administration of levamisole at 20

mg/kg body weight. The drug was administered orally to the fish foregut, and if no regurgitation occurred, the fish was used in the experiment. Six fish were taken at the following time points: 0.17, 0.5, 0.75, 1, 2, 4, 6, 8, 16, 24, 48, 72 and 144 hours after oral administration. Blood samples were obtained from the caudal vein and placed in a centrifuge tube with 1% heparin and centrifuged. The plasma supernatant was obtained. After blood was drawn, the fish were sacrificed and the muscle from two sides of the fish back bone, hepatopancreas, and kidney were excised, and all samples were stored away from light at -20°C until analysis.

*Pre-treatment of levamisole residues in plasma.*

Plasma samples were accurately absorbed and added to 15 mL centrifuge tubes. Boric acid buffer (1 mL; pH = 9) was added and then 10 mL of ethyl acetate was added. The centrifuge tube was vortexed for 2 min and centrifuged at 4000 rpm for 5 min. The supernatant was transferred to a vacuum tube of a BUCHI 48-digit multivapor. Ethyl acetate was added to the centrifuge tube, and the operation was repeated. The extracted solutions were combined, using a BUCHI 48-digit multivapor to dry the samples at 45-55°C. Methanol (200 µL) which had dried under vaporization was added to the vacuum tube and then oscillated to dissolve the sample. The samples were then analyzed using a UPLC chromatographic instrument after filtration using a 0.22 µm filter screen.

*Pre-treatment method of levamisole residue in muscle, hepatopancreas, and kidney.*

This included the following three steps.

(1) *Extract*: 5.00 g of sample was placed in a 50 mL centrifuge tube. Anhydrous sodium sulfate (5 g) was then added and mixed evenly. Boric acid buffer (1 mL; pH = 9) was then added into the centrifuge tube and vortexed for 2 min so the sample tissues were fully in contact with the buffer (Zeng, et al., 2010). 15 mL of ethyl acetate was added and vortexed for 2 min to form a paste with the tissue sample. The samples were then ultrasonicated for 10 min and centrifuged for 10 min at a speed of 4000 rpm. The organic phase was transferred into a 250 mL separatory funnel and 10 mL of ethyl acetate was added to the centrifuge tube. The operation was repeated once more to extract the remaining residues in the centrifuge tube. Then the extracts were combined with the above 250 mL in a funnel.

(2) *Liquid-liquid extraction*: 15 mL of 0.1 M hydrochloric acid solution was added to the extract and oscillated for 2~3 min to permit static settlement. The lower layer was removed to form a standby solution.

(3) *Solid phase extraction*: 3 mL of methanol and 3 mL of 0.1 M hydrochloric acid were added for activation of the mixed cation solid phase extraction column of Waters Oasis MCX. The standby solution in step (2) was passed through the column. The effluent was removed, and the column washed with 3 mL of water and 3 mL of methanol and blow dried. It was then eluted with 2 mL of methanol containing 4% ammonia water. The eluent was collected into a 2 mL glass centrifuge with scale, and then analyzed by UPLC after filtering with 0.22-µm membrane filter.

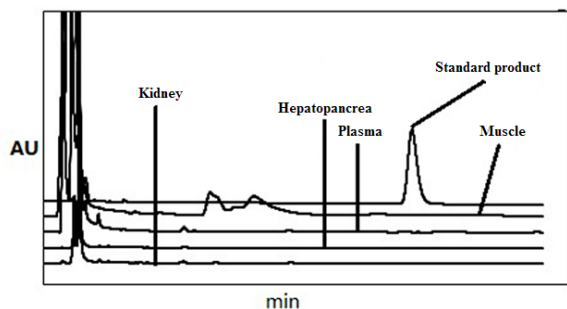
*Data analysis and liquid phase chromatographic conditions*

After pretreatment, all samples after pretreatment (plasma, muscle, viscus tissues and kidneys) were analyzed with an ultra-performance liquid chromatographic instrument-ultraviolet detector (Waters ACQUITY UPLC-UV) to obtain essential data. A Waters BEH C<sub>18</sub> (1.7 µm, 50×2.1 mm) was used as the chromatographic column, and the column temperature was maintained at 35°C. The mobile phase: 0.05 M, the ratio of phosphoric acid (adjust pH value of triethylamine to 7.0) to acetonitrile was 85:15 (V/V). The flow rate was 0.4 mL/min. The sample volume was 5 µL. The UV detection wave length was 214 nm. The results were measured by internal standard method of chromatographic and the data were processed through SPSS software. The pharmacokinetic parameters and model fitting for the essential data were assessed using DAS 3.0 software.

## Results

### Evaluation of sample test method

The method used here produced a clear levamisole peak. The peaks corresponding to plasma, muscle, hepatopancreas, and kidney impurities were well separated from the standard peak and the substance chromatographic peak in the standard sample spectrogram was spiked and symmetric. A standard sample spectrogram and the blank spectrogram can be seen in Fig.1.



**Fig. 1.** The blank and spiked standard chromatogram of muscle, hepatopancreas and plasma samples from crucian carp.

The levamisole detection working solutions were 0.1~200 µg/mL, the regression equation was  $Y=7.75e+004X+1.95e+004$  with a correlation coefficient of  $R^2 = 0.9999$ , where Y represented the chromatographic peak area and X represented the mass concentration of levamisole. The baseline noise of each blank tissue sample within the retention time of levamisole was obtained using the above analysis conditions. A triple signal-to-noise ratio was adopted as the qualitative detection limit. A decuple signal-to-noise ratio was adopted as the quantitative detection limit. The qualitative detection limit of levamisole in plasma was 0.01 µg/mL, and the quantitative detection limit was 0.03 µg/mL. The qualitative detection limit of levamisole in other tissues was 2 µg/kg, and the quantitative detection limit was 6 µg/kg.

Accuracy and precision were expressed using the recovery ratio and variable coefficient, respectively. The concentration of levamisole added to plasma was 0.03 µg/mL, 0.10 µg/mL or 0.30 µg/mL; the concentration of levamisole hydrochloride added to muscle, hepatopancreas, and kidney was 6 µg/kg, 20 µg/kg and 60 µg/kg, and the samples were tested in parallel continuously for 7 days. The recovery ratio ranged between 70% and 105%, and the variable coefficients were below 10%.

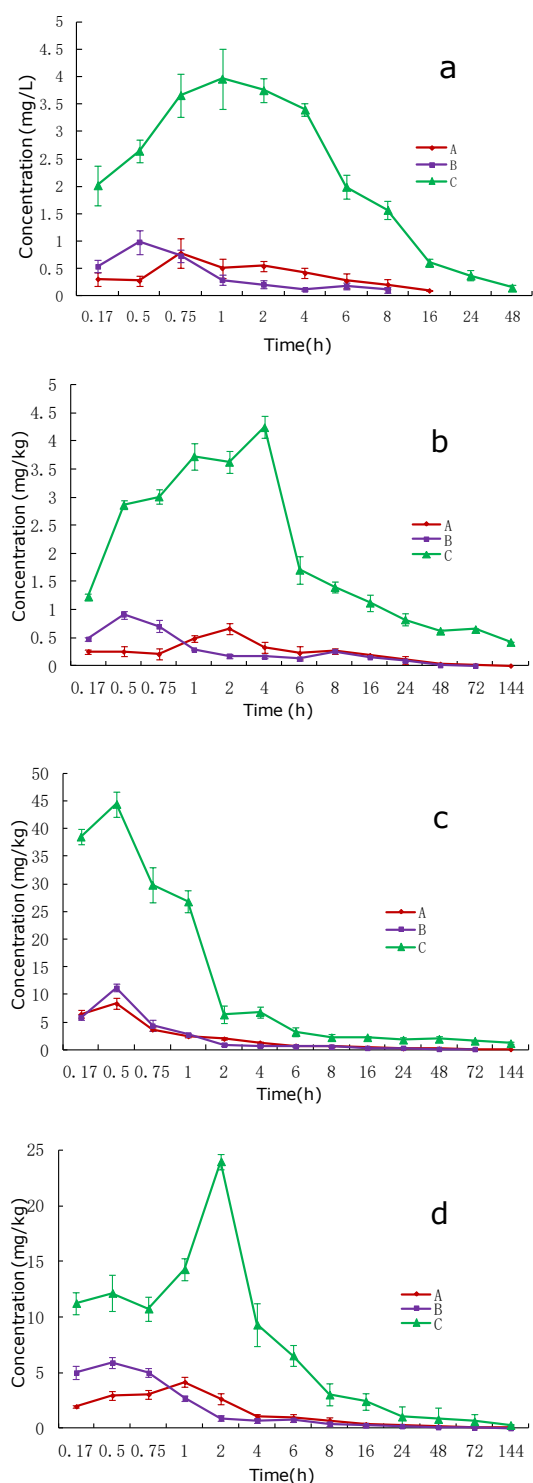
### Pharmacokinetic properties of levamisole in crucian carp

Pharmacokinetic parameters were assessed using a non-compartmental statistical moment parameters analysis on the concentration of levamisole in plasma in the three test groups using the DAS 3.0 pharmacokinetics software. The pharmacokinetic parameters are shown in Table 1 and a comparison diagram of a concentration-time curves in plasma are shown in Fig. 2a.

**Table 1:** Pharmacokinetic parameters of levamisole following oral administration to carp at different water temperatures or doses.

	Group A	Group B	Group C
Dose (mg/kg)	5	5	20
Water temperature(°C)	19 ± 1	26 ± 1	19 ± 1
Area under the curve $AUC_{(0-t)}$ (mg/L/h)	5.279	3.013	44.9
Terminal elimination half-life $t_{1/2z}$ (h)	9.927	7.051	16.559
Time to Peak $T_{max}$ (h)	0.75	0.5	1
Measured maximal concentration $C_{max}$ (mg/L)	0.785	0.987	3.965
Apparent volume of distribution $V_z$ (L/kg)	9.467	16.884	2.661
Clearance $CL_z$ (L/h/kg)	0.947	1.659	0.111

Note: for group A, water temperature was maintained at 19 ± 1°C, levamisole was administered once orally at 5 mg/kg; for group B, water temperature was t 26 ± 1°C, levamisole was administered once orally at 5 mg/kg; for group C, water temperature was 19 ± 1°C, levamisole was administered once orally at 20 mg/kg. This is true for all following Figures.



**Fig. 2.** Concentrations of levamisole in plasma (a), muscle tissues (b), hepatopancreal tissues (c), kidney tissues (d), orally-administered at different water temperatures or various doses.

#### *Absorption and distribution of levamisole in carp*

Fig. 2b, 2c and 2d demonstrate the concentration-time curves of levamisole in muscle, hepatopancreas and kidney respectively.

In all three treatment groups, levamisole concentrations in muscle tissues increased first and then decreased with time after administration (Fig. 2b). In group B at a high temperature, levamisole concentrations reached their peak of 0.91 mg/kg at a time of 0.5 h; in group A, levamisole concentrations reached their peak of 0.66 mg/kg at a time of 2 h; in group C, levamisole concentrations reached their peak last at a time of 4 h under the maximum dose, but with a peak concentration of 4.25 mg/kg.

In all three test groups, levamisole concentrations in the hepatopancreas reached their peak rapidly (0.5 h) and then decreased with increasing time after administration (Fig. 2c). Group C received the highest dose, and concentrations peaked at 44.47 mg/kg; in group B, concentrations peaked at 11.19 mg/kg; in group A, concentrations peaked at 8.34 mg/kg. This result shows that high temperature contributes to an improvement in the absorption efficiency of levamisole in the hepatopancreas.

In all three test groups, the change in the concentration of levamisole in the kidney over time was almost the same as that in muscle (Fig. 2d). Group B at high temperature, first reached a peak levamisole concentration of 5.90 mg/kg at a time of 0.5 h; group A reached a peak levamisole concentration of 4.15 mg/kg at a time of 1 h; group C received the highest dose, and concentrations peaked at 23.99 mg/kg at a time of 2 h.

## Discussion

### *Comparison of pharmacokinetic parameters of levamisole in carp plasma*

With the same dose of 5 mg/kg, administered under different water temperatures, a non-compartmental parameter analysis based on statistical moment theory using the concentration-time data of levamisole in carp plasma was performed. In the low-temperature group A at  $19 \pm 1^\circ\text{C}$  and the high-temperature group B at  $26 \pm 1^\circ\text{C}$ , the peak time  $T_{\max}$  (0.5 h) in group B was slightly shorter than that (0.75 h) in group A. The peak concentration  $C_{\max}$  of 0.987 mg/L in group B was higher than that of 0.785 mg/L in group A, which indicates that raising the water temperature can accelerate drug absorption. The elimination half-life  $t_{1/2z}$  of 7.051 h in group B was shorter than that of 9.927 h in group A, which indicates that raising the water temperature also helps drug metabolism and elimination, to shorten the elimination half-life of a drug. The area under the concentration-time curve during administration  $AUC$  of 3.013 mg/L\*h in group B was less than that of 5.279 mg/L\*h in group A, demonstrating a negative correlation with water temperature. The apparent volume of distribution  $V_z$  indicates the degree of distribution of a drug, whereby the greater the value of  $V_z$ , the wider it is distributed. The  $V_z$  of 16.884 L/kg in group B was higher than that of 9.467 L/kg in group A. The total clearance rate  $CL_z$  of 1.695 L/h/kg in group B was higher than that of 0.947 L/h/kg in group A, which indicates that  $V_z$  and  $CL_z$  have a positive correlation with temperature. In other words, as the water temperature rises, levamisole could more easily penetrate tissues and become distributed. Moreover, the drug clearance rate through the blood or plasma was also increased.

The dose is respectively 5 mg/kg (A) and 20 mg/kg (C) at a water temperature of  $19 \pm 1^\circ\text{C}$ , a non-compartmental parameter analysis was performed based on statistical moment theory using the concentration-time data of levamisole in carp plasma. The time to peak concentration of 1 h in group C was later than that of 0.75 h in group A, and the peak concentration  $C_{\max}$  of 3.965 mg/L in group C was higher than that of 0.785 mg/L in group A, which indicates that, as the dose increased, the time to peak concentration was extended, and the peak concentration increased. The elimination half-life  $t_{1/2z}$  of 16.559 h in group C was higher than that of 9.927 h in group A, which indicates that as the dose increased, the elimination half-life was prolonged. The  $AUC$  of 5.279 mg/L\*h in group A was less than that of 44.9 mg/L\*h in group C, and therefore demonstrated a positive correlation with dose. The apparent volume of distribution  $V_z$  of 2.661 L/kg in group C was less than that of 9.467 L/kg in group A. The total clearance rate  $CL_z$  of 0.111 L/h/kg was lower than that of 0.947 L/h/kg in group A. In other words,  $V_z$  and  $CL_z$  had a negative correlation with dose, which indicates that as the dose increased the amount by which levamisole penetrates tissues did not increased correspondingly.

### *Distribution and elimination of levamisole in each tissue*

The results of the current study showed that the concentration of levamisole in fish plasma, muscle, hepatopancreas, and kidney increased at first and then decreased until elimination. The concentration in the hepatopancreas peaked first, and then in the plasma, kidney, and muscle. The peak concentration in the hepatopancreas was the highest, followed by the kidney and muscle. The results demonstrate that after oral administration levamisole can be absorbed rapidly and is distributed in tissues under different conditions.

At different water temperatures, the time to achieving peak levamisole concentration in each tissue in group B was shorter than that of the corresponding tissues in group A, and the peak concentration was 1.26~1.34 times of that in group A. Levamisole was not detected in muscle and kidney at 72 h in group B, and the concentration of levamisole in the hepatopancreas was lower than the detection limit at 144 h. In group A, the concentration of levamisole in muscle and kidney was lower than the detection limit at 144 h, and at this time the concentration of levamisole in the hepatopancreas was still 0.084 mg/kg. These results indicate that the higher the water temperature the faster the levamisole was absorbed, and the greater the increase in absorption efficiency. Meanwhile, high temperature also helped in metabolizing and eliminating the drug in each tissue and reducing residues.

At the same water temperature, the dose for group C was four times more than that for group A. The time to achieving peak concentration in group C was longer than that of group A, except in the hepatopancreas. The peak concentration in group C was 2.97~5.44 times higher than that in group A, and at the same time point, the drug concentration in each tissue in group C was higher than that in group A, particularly the drug concentration in the muscle tissues, which was far higher than that in group A. The concentration in the hepatopancreas was far higher than that in the muscle.

In all three treatment groups, the concentration of levamisole in the hepatopancreas at its peak was 10.5~12.6 times more than that in the muscle, and 1.8~2.0 times more than that in the kidney. The residence time of levamisole was longest in the hepatopancreas, and therefore the hepatopancreas has been identified as the residual target tissue of levamisole in carp. In addition, the drug concentration in the kidney was far higher than in the muscle and plasma, and only lower than that in the hepatopancreas. Therefore, the kidney has also been identified as a major organ for metabolizing levamisole.

### Acknowledgements

This work was supported by the natural science foundation project of Jilin province (No.20140101035JC) and national modern agriculture industry technology system - the construction project(CARS-45-35).

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