

Chloramine-T Concentrations in Serum and Tissue of Tilapia Fish After immersion Therapy in Two Different Concentrations of Chloramine-T

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ABSTRACT

Chloramine-T (Cl-T), a chlorine-liberating organic compound with a biocidal effect, is applied for several purposes such as drinking water disinfection or as an antiseptic for topical infections. The standard treatment regimen is to apply Cl-T at a concentration of 10 or 20 mg/L for 1.0 hours/day for 3-4 consecutive days. The planned goal of the current work was to apply Cl-T by immersion in a static bath continuously for 3 days, and then evaluate its residual concentrations in serum and edible fish tissues. A total number of 65 tilapia (*Oreochromis niloticus*) fish were used in the current study: 5 fish samples were used for calibration curve preparation while the other 60 fish individuals were divided into 2 equal groups; the first was exposed to 10 and the second was exposed to 20 mg Cl-T/L; each exposure persisted for 3 consecutive days at 20°C. Chloramine concentrations were assayed in serum and edible tissue (muscles, liver and kidney) of tilapia for 5 days post-treatment with Cl-T using HPTLC (High-performance thin layer chromatography). No signs of toxicity or mortalities were recorded in tilapia exposed to either 10 or 20mg Cl-T/L in such a regimen. Six hours after the end of the low dose (10mg/ L) of Cl-T dipping, muscle tissues retained the highest chloramine residual levels (149.74±17.52 µg/g) compared to liver and serum (0.84±0.13 µg/g and 1.62±0.06 µg/mL, respectively); meanwhile, kidney samples were under our detection limit (<11.25 ng). Similarly, 6 hours after the end of exposure to 20mg Cl-T/L, muscles recorded the highest concentration of chloramine residues (322.12 ± 34.79 µg/g) compared to liver and serum (2.36 ± 0.30 µg/g and 3.68 ± 0.14 µg/mL, respectively). The Cl-T residues could be detected in muscles up to 4 days post the end of Cl-T dipping by such practice. Further studies are needed to calculate the Cl-T withdrawal time after such a long immersion protocol.

INTRODUCTION

Chloramine-T (Cl-T, sodium p-toluenesulphonchloramide) is an external disinfectant that was lately approved by the U.S. Food and Drug Administration (FDA) for its therapeutic uses in aquaculture to reduce mortality related to external bacterial infections and bacterial gill disease in aquaculture (Bowker *et al.*, 2011). It can be applied as static

or flush treatments during periods of disease risk or in response to both pathogen detection and obvious disease symptoms (**Quezada-Rodriguez *et al.*, 2022**).

As a strong oxidizer, Cl-T has many advantages in aquaculture, the most important are: its low accumulation in the living organisms and its very slow microbial (bacterial or parasitic) resistance development, as microorganisms are theoretically unable to develop tolerance to oxidation (**Bowker *et al.*, 2011**).

In practice, the dose and treatment strategy may vary according to the disease affecting the fish population. In fish farming, Cl-T are used to prevent and control bacterial gill disease, for preventive purposes, 10mg Cl-T /L of basin water for an hour is the common treatment procedure (this process may be repeated every 15 to 30 days); while for therapeutic purposes, the same concentration and duration are recommended but usually repeated up to three times in a week. (**EMEA, 2005**).

Treatment of food producing animals such as fish with disinfectants is an important step to reduce micro-organisms threatening and to prolong the shelf-life of animal-origin food products. Such uses of disinfectants are only permitted when they become registered according to the provisions of national regulations that ensure the safety of remaining residues in the food of consumers. Such practices are also applied in the fish industry (**Dvorak, 2008; Nikhanj *et al.*, 2022**).

While there are numerous studies on the efficacy and the margin-of-safety of Cl-T in fishes using up to 5-times the highest proposed dose for up to 3-times the proposed treatment duration, only scanty experiments were performed to evaluate the Cl-T depletion or its residues in fish.

Therefore, the present study was conducted to evaluate the tilapia survival and quantify the Cl-T residues in tilapia tissues using an unusual treatment regimen. To achieve this goal, two concentrations of Cl-T (10 and 20 mg/L) were applied by dipping for three successive days to healthy tilapia fish.

MATERIAL AND METHODS

Drug and chemicals

Chloramine-T used in the current study (Halamid[®], Axcentive Sarl, Bouc-Bel-Air, France) was obtained from local market. HPTLC silica gel 60F254 plates (Merck Ltd., USA), toluene, ethyl acetate, potassium ferrocyanide, zinc acetate, sodium sulfate (Loba Chemie Pvt.Ltd., India) and other chemicals were of HPLC grade with purity of at least 99.9% (**Rondags *et al.*, 1978**).

Experimental animals

A total number of 60 healthy tilapia fish (*Oreochromis niloticus*) with an average body weight of 120±10g were obtained from El Manzala Lake, Egypt (brackish water) and

Chloramine-T in Serum and Tissue of Tilapia After immersion Therapy in Two Different Concentrations of Chloramine-T

transported alive to the laboratory of Pharmacology Faculty of Veterinary Medicine Suez Canal University, Ismailia, Egypt.

Study design and fish grouping

Five tilapia fish were kept untreated and used for standard curve preparation, while the other sixty fish were divided into 2 equal groups, each of which was kept in six glass tanks with dimensions 50 × 50 × 100cm. Fish tanks were filled with dechlorinated tap water and provided with aerating devices and water filter. Water dissolved oxygen ranged from 6.5 to 8mg/ L, the average water pH was 7.2, while water temperature was maintained between 19 and 20°C. Chloramine was added to the first group's (30 fish) tanks at a concentration of 10mg/ L, and at 20mg/ L for the second group's (30 fish) tanks; chloramine immersion was applied for 3 consecutive days for both groups.

Sampling

At each time point (6, 24, 48, 72, 96, and 120 hours post chloramine application), blood (collected from the caudal vein to obtain serum) and tissues (muscle, liver, and kidney) samples were taken from 5 fishes. Samples were kept in deep freezer at -20°C till assessing the levels of chloramine residues at Central Laboratory of Veterinary Medicine Faculty, Suez Canal University.

Analytical method

Chloramine-T was extracted from serum and tissues using homogenizer and 2.5mL distilled water, 2.5 potassium ferrocyanide, 2.5mL zinc acetate and 1.5mL sodium sulfate, following the method described by **Rondags *et al.* (1978)**. Chloramine-T was converted to p-TSA in a deproteinized sample. The p-TSA was isolated from the sample solution by extraction, separated by TLC, and determined densitometrically by *in situ* reflectance measurements. HPTLC quantitative technique was performed based on the method described by **Meinertz *et al.* (1999)**. Separation was carried out using 20 cm × 10 cm HPTLC silica gel 60F254 plates (Merck), with mobile phase consisting of toluene: ethyl acetate in 70:30 ratio.

Sample application

Stock solutions containing 500µg/ mL of chloramine were prepared in ethyl acetate. Working standard solutions containing serial concentrations (12.5 ng - 200 µg/mL) of chloramine were prepared by suitable dilution of the stock solutions with ethyl acetate. All samples and standards were applied to the plates by means of CAMAG Linomat 5 with dosing syringe of 100µL as 7 mm bands with 10.5 mm Distance between tracks was determined, with an application × 15 mm and 15 mm application Y edges of plate, and the application volume was 5 µL for samples and standard.

Chromatogram development

The HPTLC plates were developed in CAMAG Automatic Developing Chamber at room temperature ($25^{\circ}\text{C} \pm 5^{\circ}\text{C}$). The development occurred in a two-step procedure (preconditioning with 10mL mobile phase for 5 minutes and development with 25mL mobile phase for 20 minutes). The plate was developed to a distance of 70mm and dried for 5min by a stream of warm air.

Detection

The plates were scanned and examined densitometry at 1- 5nm by means of CAMAG TLC Scanner 4, with slit dimension of $6 \times 0.30\text{mm}$ at multi wavelength of 228nm.

The chromatogram is evaluated under white or UV light. All the functions of the scanner were controlled by the software Win CATS. Only the positioning of the objects to be scanned was performed manually.

Statistical analysis

Calculation was done by means of statistical software package entitled Mini Tab[®] (version 17, 2010) and least significant was redeemed to be at $P \leq 0.05$. Data were tabulated and statically analyzed to evaluate the difference between groups in regard to the various parameters (**David *et al.*, 2003**). The arithmetic mean, standard error and one-way analysis of variance (ANOVA) were tabulated.

RESULTS

Chloramine-T safety

No mortalities or signs of toxicity were recorded in tilapia exposed to either 10 or 20mg/L concentrations by immersion for 3 continuous successive days.

Chloramine calibration curves in serum and tissues

The best absorbance wavelength and retention factor (RF) were confirmed from all track's absorbance scanning as shown in Table (1) and Fig. (1). The standard curve linearity was cofirmed up to $100\mu\text{g}/\text{mL}$ serum or g of tissues, with a coefficient of correlation (r^2) > 0.99 in serum and >0.98 in tissues. The recovery percentage was 92.1, 73.2 and 68.1% in serum, muscles and liver, respectively, as shown in Fig. (2).

Chloramine-T in Serum and Tissue of Tilapia After immersion Therapy in Two Different Concentrations of Chloramine-T

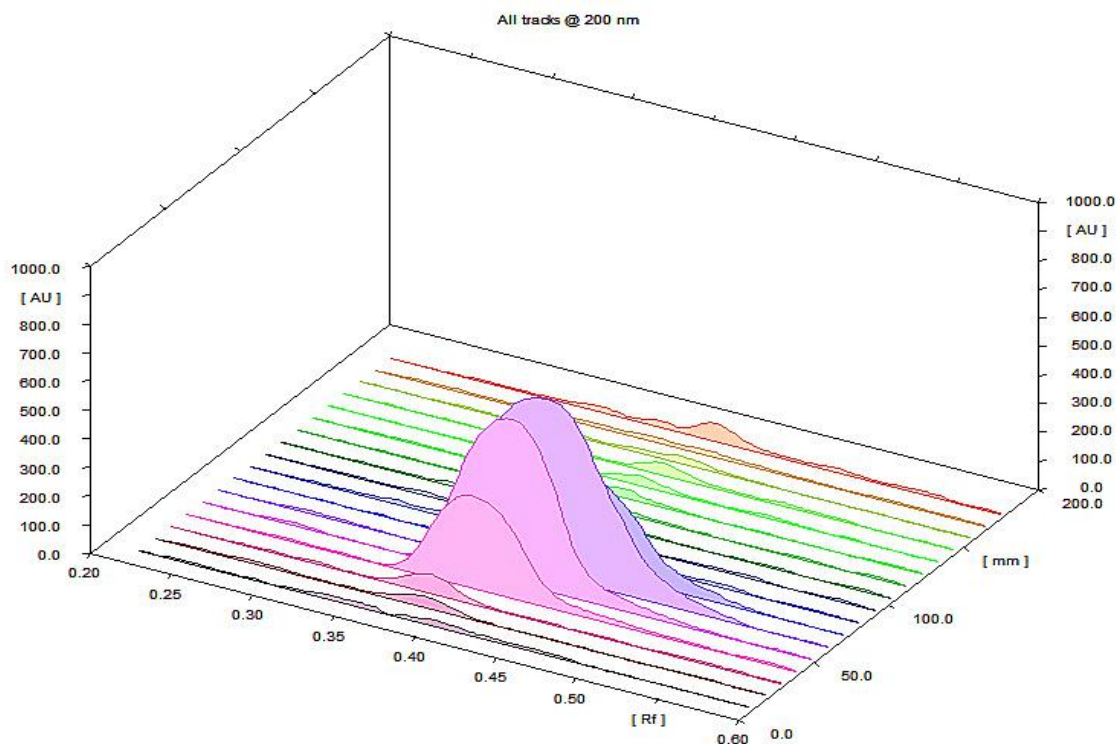


Fig. 1. A multi-track scanning of chloramine using HPTLC, the scan viewing the best absorbance wavelength (228nm) and the best RF time (0.30- 0.35)

Chloramine-Residues in serum, muscle, liver and kidney at dose 10mg/ L

Six hours after chloramine administration, the highest concentration was observed in muscles compared with serum and liver, while kidney was under detection limit (<11.25 ng) throughout the experimental period. Starting from the 1st day post administration, the liver's concentrations exceeded those measured in serum. The chloramine residues decreased continuously with time till the 4th day, where only one fish showed a very low concentration of chloramine residue in muscle. Meanwhile, other organs were under the detection limit. By the 5th day post chloramine exposure, no residues could be detected in all fish serum or tested tissues (Table 2).

Chloramine-Residues in serum, muscle, liver and kidney at dose 20mg/ L

Similar to the low dose results, the highest chloramine residues were observed in muscles ($322.12 \pm 34.79 \mu\text{g/g}$), and the lowest was spotted in liver; meanwhile, the kidney was under the detection limit (<11.25 ng). At the 4th day post chloramine-T exposure, the chloramine could be detected only in all fishes' muscles at very low concentrations. Five days post exposure, chloramine-T residues were under detection limit in all assayed samples, except for only one muscle sample that measured $0.02 \mu\text{g/g}$ (Table 3).

Table 1. Comparative standard curves of chloramine in fish's serum and tissues after being spiked with serial known concentrations

| Chloramine concentrations ($\mu\text{g/mL}$ or g) | Corresponding absorbance AUC values | | |
|---|-------------------------------------|---------|----------------|
| | Serum | Muscles | Liver & kidney |
| 0.125 | 505.8 | 421.5 | 392.9 |
| 0.4 | 1018.5 | 948.7 | 882.3 |
| 50 | 13492.7 | 11543.9 | 10735.8 |
| 100 | 26704.8 | 21254.3 | 19766.5 |

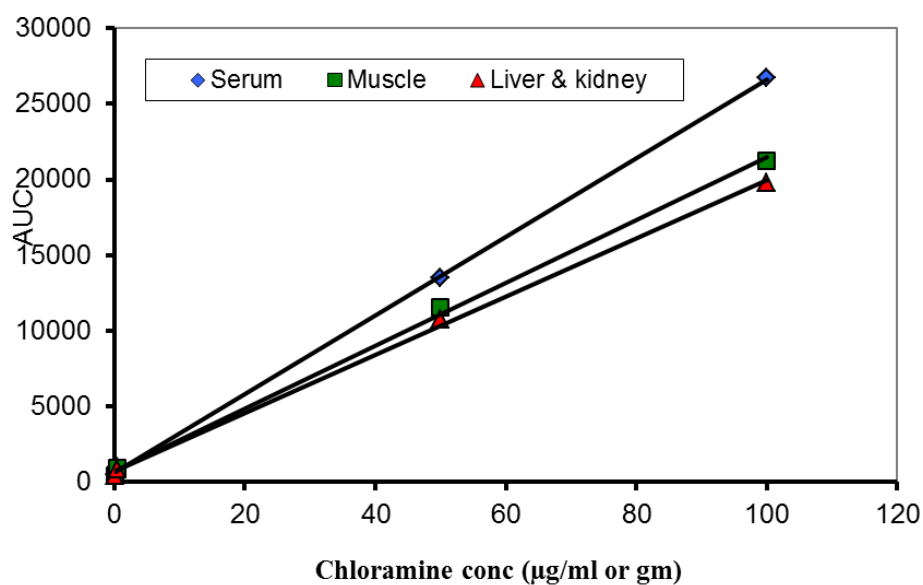


Fig. 2. Comparison between standard curves of chloramine in fish's serum and tissues

Chloramine-T in Serum and Tissue of Tilapia After immersion Therapy in Two Different Concentrations of Chloramine-T

Table 2. Chloramine-T concentrations in fish serum & tissues 5 days after exposure to chloramine-T at a dose of 10mg/ L for 3 successive days

| Day post treatment | Concentration ($\mu\text{g/mL}$ or g) | | | |
|--------------------|--|----------------------|-------------------|-----------|
| | Serum | Muscle | Liver | Kidney |
| 0.25 | 1.62 ± 0.06^b | 149.74 ± 17.52^a | 0.84 ± 0.13^b | <11.25 ng |
| 1 | 0.14 ± 0.03^b | 30.11 ± 7.39^a | 0.20 ± 0.05^b | <11.25 ng |
| 2 | 0.01 ± 0.00^b | 2.34 ± 0.48^a | 0.02 ± 0.01^b | <11.25 ng |
| 3 | <11.25 ng | 0.16 ± 0.08^a | <11.25 ng | <11.25 ng |
| 4 | <11.25 ng | 0.004 ± 0.004^a | <11.25 ng | <11.25 ng |
| 5 | <11.25 ng | <11.25 ng | <11.25 ng | <11.25 ng |

Means within the same row with different superscripts are significantly different ($P < 0.05$).

Table 3. Chloramine-T concentrations in fish serum & tissues 5 days after exposure to Chloramine-T at a dose of 20mg/ L for 3 successive days

| Day post treatment | Concentration ($\mu\text{g/mL}$ or g) | | | |
|--------------------|--|----------------------|-------------------|-----------|
| | Serum | Muscles | Liver | Kidney |
| 0.25 | 3.68 ± 0.14^b | 322.12 ± 34.79^a | 2.36 ± 0.30^b | <11.25 ng |
| 1 | 0.38 ± 0.07^b | 81.40 ± 17.18^a | 0.85 ± 0.20^b | <11.25 ng |
| 2 | 0.04 ± 0.02^b | 12.48 ± 1.86^a | 0.19 ± 0.05^b | <11.25 ng |
| 3 | <11.25 ng | 1.57 ± 0.47^a | <11.25 ng | <11.25 ng |
| 4 | <11.25 ng | 0.19 ± 0.05^a | <11.25 ng | <11.25 ng |
| 5 | <11.25 ng | <11.25 ng | <11.25 ng | <11.25 ng |

Means in the same row carrying different superscript letters are significantly different at $P < 0.05$

DISCUSSION

The present study was undertaken to evaluate the safety of Cl-T applied by immersion for 3 successive days in tilapia fish and quantify the Cl-T residues in healthy fish after Cl-T application in such manner. To our knowledge, there are no previous published investigation about chloramine-T residues in tilapia species.

Indeed, Cl-T was not applied by immersion in static bath for 3 continuous days for tilapia or any type of fish before. The fact that inspired us to use such easier and more reliable immersion protocol was the limited Cl-T absorption from water in fish. This fact was evidently stated by **Mitchell et al. (2000)** who reported that, chloramine-T was poorly

absorbed from water when trout fish was immersed for one hour in radiolabeled chloramine-T (20 mg/L).

In our presented work, no signs of toxicity or mortalities were observed in tilapia exposed to either 10 or 20mg/ L concentrations for continuous 3 successive days.

The recovery percentages in our study were 92.1, 73.2 and 68.1% in serum, muscles and liver, respectively. The linearity of the calibration curve was confirmed up to 100µg/ mL serum or g of tissues with a coefficient of correlation (r^2) > 0.98. The total residue depletion and metabolism of Cl-T (Halamid[®]) as well the validation the methods used to analyze p-TSA in edible tissue of cold-water fish (rainbow trout) was accepted by **FDA, CVM (Schnick *et al.*, 2000)**.

At the 10mg chloramine-T / L concentration, the muscles tissues retained the highest concentration of chloramine residue ($149.74 \pm 17.52\mu\text{g/g}$), compared to liver and serum ($0.84 \pm 0.13\mu\text{g/g}$ and $1.62 \pm 0.06\mu\text{g/mL}$), respectively; meanwhile, kidney samples were under detection limit (<11.25ng).

Similarly, exposure to 20mg chloramine-T / L resulted in even higher concentrations in muscles ($322.12 \pm 34.79\mu\text{g/g}$) compared to liver and serum ($2.36 \pm 0.30 \mu\text{g/g}$ and $3.68 \pm 0.14 \mu\text{g/mL}$) respectively. Still kidney samples showed no residues of Cl-T (our detection limit was 11.25 ng).

These concentrations in the present study are much higher than those reported by other investigators such as **Meinertz *et al.* (2004)** who assayed muscle concentrations of 142ng/ g in striped bass, 97ng/ g in rainbow trout, and 150ng/ g in yellow perch immediately after Cl-T exposure (20mg/ l of chloramine-T for 60min on 4 consecutive days). In a field study on around 700 diseased fish (cultivated in a 405m² pond) that were treated with chloramine-T (2 ppm = 20 mg/L), residue analysis displayed a range of 46.13 to 60.54 ng p-TSA /g tissue (**Mitchell, 2000**).

The very high Cl-T residual levels in our investigation compared to other's results could be basically assigned to 2 factors; the long immersion time in our study (3 continuous days) compared with the short immersion time in other's investigations (1.0 hour/day for 3-4 successive days) and the water temperature differences (19-20 °C in the current study contrasted to 7-15 °C in other's experiments). Effect of water temperature on Cl-T toxicity and chlorine liberation rate was confirmed during the past several decades (**Bills *et al.*, 1988**). Additionally, numerous researchers including (**Sohlberg *et al.*, 1994**) and (**Khalil *et al.*, 2016**) have extensively investigated and supported the impact of temperature on the absorption and excretion of various antimicrobials in aquaculture.

CONCLUSION

It can be concluded that, chloramine-T can be used by immersion in static bath for 3 continuous days for tilapia without mortalities or signs of toxicity, neither for 10 nor for 20 mg Cl-T/L concentrations. Under such long-time immersion protocol, the Cl-T residual

Chloramine-T in Serum and Tissue of Tilapia After immersion Therapy in Two Different Concentrations of Chloramine-T

concentrations were high in muscles and persisted for 3- 4 days post exposure. Further studies are critically needed to calculate the Cl-T withdrawal time after such long-time immersion protocol.

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