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Research Article

Studies of Formalin Application and Drug Residue Analysis in the Cultured Hybrid Tilapia, *Oreochromis Hybrids* by High Performance Liquid Chromatography with Ultraviolet Detection

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2. Keywords

Formalin; High performance liquid chromatography; Pharmacokinetics; Tilapia; Ultraviolet detection

3. Abbreviations: C.V.: Coefficient of variation; HPLC: High-performance liquid chromatography; IACUC: Institutional animal care and utilization committee; LOD: Limit of detection; LOQ: Limit of quantification; p.o.: Oral administration; S.D.: Standard deviation; UV:Ultraviolet

1. Abstract

Formalin (37% formaldehyde) is approved for use in aquaculture as a chemical for controlling ectoparasites and aquatic fungi in the USA, Canada, and Asia while it is prohibited in Australia, Europe, and Japan because of its association with oncogenesis. Safety about formalin-treated aquatic animals used for food has been discussed in the aquaculture industry. The aim of this study is to determine the residual concentrations of formalin in muscle and liver in cultured tilapia (*Oreochromis hybrids*) by high performance liquid chromatography with ultraviolet detection. Acute toxicity of formalin to tilapia was determined under a semi-static condition at 27°C. Tilapia bathed with formalin at the single concentration of 25 and 50 ppm, respectively. Our results showed that the median lethal concentration (LC_{50}) lowered progressively as the duration of exposure increased. The toxicity curves became almost asymptotic with the time axis by 96 h-formalin exposure. The 48 h-LC_{50} and 96 h-LC_{50} was 179 and 177.2 ppm at 27°C, respectively. Under 50 ppm formalin treatment, gill lamellar epithelia separation, hypertrophy, and necrosis under the histopathology examination were shown. Meanwhile, the time of undetectable residue amount of formalin in tilapia liver at 25 and 50 ppm was 0 and 2 h and in muscle was 2 and 2 h, respectively. The recommendation of formalin can be used at a concentration of 25 and 50 ppm in tilapia without resulting in formaldehyde residues in the fish edibletissues.

4. Introduction

Formalin was used for controlling protozoan parasites of fish [1-2]. Applications of formalin were used in the industrial applications, disinfectant and biocide, tissue fixative and embalming agent, drug testing, and Hobbies etc [2]. Formaldehyde is highly toxic to all animals, regardless of method of intake. Ingestion of as little as 30 mL (1 oz.) of a solution containing 37% formaldehyde has been reported to cause death in an adult human [3]. Water solution of formaldehyde is very corrosive and its ingestion can cause severe injury to the upper gastrointestinal tract. Moreover in view of its widespread use, toxicity and volatility, exposure to formalin is a significant consideration for human health [3].

Tilapia (*Oreochromis sp.*) is the second most important group of farmed fish after carps, and the most widely grown of any farmed fish. Tilapia culture and industry have become one of the most important issues worldwide. According to the record of Food and Agriculture Organization of the United Nations, Nile tilapia (*Oreochromis niloticus*) production was gradually increased in the world from 1,590 tons in 1950 to 2,790,350 in 2011 [4]. Tilapia culture and export to the world market is an important

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industry in Taiwan. The usually high density of animals grown in agricultural facilities and fish hatcheries can increase the potential for disease outbreak. With development of the intensive aquaculture industry, large amounts of antibiotics are extensively used in tilapia farms to prevent and therapy pathogen infections [4]. Thus, an increase in drug-resistant strains of bacteria and the possibility that drug residues in tilapia could cause public hygiene and health problems [5]. However, there have been no reports on the pharmacokinetics of formalin in the cultured hybrid tilapia, *Oreochromis hybrids*. Thus, the study deals with the pharmacokinetics and drug-resistant of formalin after administration in cultured hybrid tilapia.

Sanitizers are commonly used in aquaculture for disease prevention. Among these drugs, formaldehyde is an effective sanitizer against pathogens and has been widely used in fish culture [4, 6-7]. However, only a handful of known concentrations of formaldehyde are available to be used effectively and without damage to fish [4]. Thus, pharmacokinetic investigations are important for defining the dosage regimens of formalin. In tilapia, formalin is demonstrated to have immunosuppressive effects [3] and reproductive toxicity [4]. While in human, mice, and rats, it has reproductive toxicity [8-10]. In order adequately to protect the consumer and secure dairy aquacultural productions, formalin is a non-legal aquatic animal drug and is not detected in fish tissues based on the Standards of Veterinary Drug Residues in Aquatic Animal Drugs Use in Taiwan and Japan. In China, formalin is a widely used sanitizer in aquaculture, while the appropriate concentration and side effects is not available to be used effectively and without damage to tilapia [4]. At present, three brands of formalin, FORMALIN-F (Natchez Animal Supply, Natchez, MS, USA), PARACIDE-F (Argent Chemical Laboratories, Redmond, WA, USA), and PARASITE-S (Western Chemical Inc., WA, USA), have been approved by FDA, USA as parasiticides for use on fish. Thus, a proper withdrawal period for formalin is needed after the drug administration in veterinary medicine for ensuring that drug residues in edible tissues are below established tolerance levels.

At present, drug residues caused drug resistance for pathogens has become a global threat. Drug residues in aquatic animal tissues pose a potential threat to human health. There is an obvious need for rapid and accurate methods for measuring drug residues in edible animal tissues to protect consumers' health [11]. Additionally, pharmacokinetic profiles of formalin were not investigated in hybrid tilapia, an *Oreochromis hybrid which is* commercially important fish species in Taiwan and world. Based on the pharmacokinetic studies and the withdrawal period of formalin in tilapia is lacking, therefore, development of a simple, rapid, and reliable technique to detect the pharmacokinetics of formalin will help researchers to understand formalin metabolism in hybrid tilapia. Thus, the objectives of this study were to determine the pharmacokinetic parameters of formalin in the hybrid tilapia following single-dose bath administration. Based on the pharmacokinetics of formalin in hybrid tilapia, it will be applied to the prevention of infectious diseases in clinical. The trial was the first study to investigate the pharmacokinetics of formalin before and after various periods of administration in farming tilapia using high performance liquid chromatography with ultraviolet detection. These data will be as a reference basis for withdrawal period of formalin in farming tilapia in Taiwan and world.

5. Materials and Methods

5.1. Chemicals

In this study, HPLC grade methanol and acetonitrile were purchased from Mallinckrodt Baker (Phillipsburg, NJ, USA). Formalin (CH_2O; 37% assay purity) was purchased from Union Chemical works Ltd (Hsinchu City, Taiwan) (Figure 1). Formalin was dissolved in doubly deionized water at the stock concentration of 1,000 ppm and stocked at -20°C. The stock solution of formalin was diluted with doubly deionized water to 150, 160, 170, 180, 190, 200, 250, 300, 400, 500 ppm, respectively until the beginning of the experiments. Other analytical grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

5.2. Apparatus

The HPLC system consisted of a quaternary solvent delivery system (model 600e) (Waters, Milford, MA, USA), an autosampler (model 717, Waters), a photodiode array detector (model 996, Waters), and a Cosmosil $5C_{18}$-MS column (5 µm, 4.6 × 150 mm i.d.; Nacalai, Kyoto, Japan). The ultraviolet detector (model 2475, Waters) with auto-change wavelength and the wavelength of 360 nm was used in this study. Analysis of HPLC data was performed with Millennium 32 software (Version 4.0, Waters).

5.3. Animal Care

All animal experiments were approved by the Institutional Animal Care and Utilization Committee (IACUC) of National Chung-Hsing University, Taichung, Taiwan (approval ID: 95-050), and animal care was performed in compliance with the guidelines of IACUC and the United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

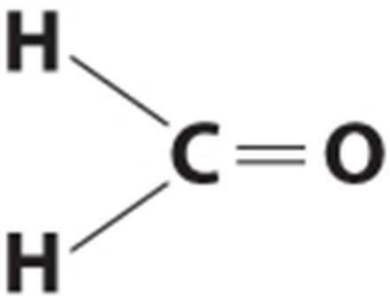

Figure 1: The chemical structure of formaldehyde.

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Healthy Tilapia, a cultured *Oreochromis hybrids* (body weight: 10 ± 0.6 g and 500-600g) were respectively obtained from Fisheries Research Institute, COA (Changhua county, Taiwan) and a cultural tilapia farm (Syuejia District, Tainan City, Taiwan). Large-size fishes were held in our laboratory fish ponds with UV filtered running aerated fresh water (27 ± 1°C) and small-size fishes were housed in the 270 L experimental tank (length: 120 cm × width: 60 cm × depth: 45 cm) with a running and UV filtered re-circulating aerated fresh water (Temperature: 27°C, pH: 8.1-8.5, and dissolved O_2: 6-6.3 ppm) in the Central Fish Disease Center, National Chung-Hsing University, Taichung, Taiwan. Experiments began after an acclimatization period of at least 1 month to overcome the effects of transportation stress. Fish were fed twice daily with tilapia pellets purchased from the feed factory (FWUSOW industry Co., Ltd, Tainan, Taiwan) and kept on a 12-h light/dark cycle. Composition of the tilapia pellets included 52.38% crude protein, 8.22% crude fat, 10.03% ash content, 5.26% calcium, 2.98% phosphorous, 0.14% sodium, 0.72% magnesium, and 20% starch. Before the experiment, the muscle and liver of tilapia were obtained from ten fishes and identified no residues of formalin in these samples.

5.4. Experimental Design and SampleCollection

First, the organs were collected, histopathologic examination, and mortalities were recorded from ten small-size fishes per group after bathing with the various doses of formalin (150, 160, 170, 180, 190, 200, 250, 300, 400, 500 ppm) for 2, 4, 6, 24, 48, 72, and 96 h. Additionally, 30 large-size fishes/group bathed with 25 and 50 ppm formalin, respectively. The age- and size-matched control fish bathed with fresh water without formalin. After bathing with/without formalin for 1, 2, 4, 8, 24, 48, 72, and 96 h, six fishes/group were catch randomly for detection of formalin residues in skin, muscle, and liver.

5.5. Histopathologic Examination

After bathing in the various concentrations of formalin, fish were humanely euthanized with tricaine methanesulfonate (MS222; Sigma-Aldrich). Gills, liver, skin, muscle, and eyeball samples were asepticallytaken a sample and submitted for histopathologic examination. Tissues were dehydrated by serial ethanol and xylene stages for automated processing to wax, and 5 μm sections were examined. All of the slides were stained with hematoxylin and eosin (HE) (BBC Biochemical, USA) and observed microscopically.

5.6. Sample Pretreatment and Derivatization

Respectively, 2 g muscle and 2 g liver of tilapia were immediately well mixed with 10 mL extraction solution [the mixture of 1.0 N NaOH (64.3 mL) and glacial acetic acid (5.7 mL) in 930 ml doubly deionized water; pH 4.93 ± 0.02]. Later, each mixture was homogenized by homogenizer (OMNIMacro ES, NY, NY, USA) at 10,000 rpm for 1.5 min and ultra-sonication for 10 min. Finally, centrifugation was at 6,000 rpm for 10 min to collect the supernatants and sediments. The sediments were immediately well-mixed 10 mL extraction solution and followed the above procedures to collect the supernatants again. Later, these supernatants of muscle and liver well-mixed with 6 mL 2,4-dinitrophenylhydrazine (3 g/L) and 4 mL acetate buffered solution on a water bath (Bransonic 8210, Branson Inc., Danbury, CT, USA) for 60 rpm, 60°C, and 20 min. Finally, cool to 20°C in cold water and cool mixture was well mixed with 10 mL saturated $NaCl_{(aq)}$ and filter through 0.45 μm PVDF syringe filter (Acrodisc, Pall Inc., NY, NY, USA) until HPLC analysis. Additionally, culture water was also collected at these several time points of formalin bath to detect the volatilization rate of formalin in culture water following the same procedure of muscle and liver derivatization.

5.7. Sample Extraction and Clean-up

Formalin extraction was performed by solid phase extraction (SPE). The sample extraction and clean-up of formalin was carried out according to previously described procedures [12].

5.8. Derivatization

The extracted and clean-up sample (0.1 mL) was well-mixed with 20% TCA (100 μL) for 5 sec, then immediately well-mixed with 200 μL 7% formaldehyde (Merk, Darmstadt, Germany). Later, to add DDW to 1 mL of total volume and incubate at 100°C for 30 min. Finally, cool to 20°C in cold water and filter through 0.2 μm PVDF syringe filter (Acrodisc, Pall Inc., NY, NY, USA) until HPLC analysis.

5.9. Method Validation

Linearity was assay according to the slopes, intercepts, and correlation coefficients of the calibration curves were calculated by linear regression analysis. The recovery was determined by repetitively analyzing blank muscle (2 g) and liver (2 g) supplemented with/without known amounts of formalin (20 ppm). After derivatization, the recoveries of formalin were performed by using HPLC with ultraviolet detection. Calculation of recoveries was following as: Recovery = (the eluted formalin concentration in samples / the accurate adjunction of formalin concentration in samples) × 100%.

5.10. Limit of Detection and Limit ofQuantification

The limit of detection (LOD) and limit of quantification (LOQ) were estimated from the size of the formalin peak in spiked samples of blank muscle or blank liver.

5.11. Stability of Formalin in Extracts

The peak areas of formalin and the internal standard in extracts were determined at time zero and after 24 h of storage at room temperature.

5.12. Determination of Formalin

Samples (20 µL) were analysed on a Watersquaternarysolvent deliverysystem (model 600e) (Waters, Milford, MA, USA). Separation was achieved on Cosmosil $5C_{18}$-MS column (5 µm, 4.6 × 150 mm i.d.; Nacalai, Kyoto, Japan) and equilibrated with 3 mL deionized water/2 mL, 2% acetonitrile (80/20, 70/30, 50/50, or 40/60, v/v) at a flow-rate of 1 mL/min for 1 h in HPLC system (Waters). After all samples were passed through the column, the solution of 3 mL deionized water-2 mL, 2% acetonitrile was used for washing. Finally, 5 mL acetonitrile (HPLC grade, Merk, Darmstadt, Germany) was used for rinse formalin conjugated in the SPE and Cosmosil $5C_{18}$-MS column at a lower flow-rate of 1 mL/min. The total volume of rinse was 5 mL. The ultraviolet detector (model 2475, Waters) with the wavelength of 360 nm was used. Analysis of HPLC data was performed with Millennium 32 software (Version 4.0, Waters).

5.13. Assay Validation

The intra-assay and inter-assay of formalin was carried out according to previously described procedures [12]. The concentration of formalin in samples was determined using the linear regression line (unweighted) of the concentration standard versus peak area ($r^2 = 0.9957$). The precision of the method was expressed as the intra-day and inter-day coefficients of variation (%), which were assayed (three replicates) at concentrations of 0.2, 2, and 20 ppm of formalin on the same day and on three sequential days, respectively. The accuracy (% bias) was calculated from the nominal concentrations (C_{nom}) and the mean value of the observed concentrations (C_{obs}) as follows: bias(%) = $[(C_{obs} - C_{nom}) / (C_{nom})] \times 100$. Therelative standard deviation (RSD) was calculated from the observed concentrations as follows: precision (% RSD) = [standard deviation (SD) / C_{obs}] × 100. Accuracy and precision values within ± 20% covering the actual range of experimental concentrations were considered acceptable. Additionally, instrumental precision was determined as described [12]. Three concentrations of formalin (0.2, 2, and 20 ppm) were analyzed three times on the same day for 3 days to determine within-run precision. The fluctuation of the slopes of the calibration curves was an indication of the day-to-day precision.

6. Statistical Analysis

In this study, data values are reported as mean ± the standard deviation (S.D.). Statistical evaluation was performed using Student's *t*-test. Differences between groups were considered statistically significant at $^{*}p < 0.05$.

7. Results

7.1. Acute Toxicity of Formalin in Tilapia

Acute toxicity assay was performed in tilapia after the several time points (2, 4, 6, 24, 48, 72, and 96 h) of formalin (150, 160, 170, 180, 190, 200, 250, 300, 400, or 500 ppm) exposure (bath treatment) to evaluate clinical behavior and mortality of tilapia. Each group (n = 10 fishes) bathed with formalin for several time points to calculate 50% lethal dose (LD_{50}) of formalin and 95% confidence interval within 96 h-formalin bath. Under 400 and 500 ppm formalin bathing, all of 20 fishes successively died within 1 day of formalin bath. In addition, under 200, 250, and 300 ppm formalin bath, all of 60 fishes died within 2 day of formalin bath. Fortunately, under 150 ppm formalin bath, all fishes were survival. Between 200-500 ppm formalin bath, fish occurred sudden death and 100% mortality was presented in tilapia. However, at 150 ppm formalin bath, all fishes were survival. Additionally, 48 h-LD_{50} and 96 h-LD_{50} were respectively 179 and 177.2 ppm (data not shown). Clinical examination of the tilapia with formalin (200, 250, 300, 400 or 500 ppm) bath within 1-2 h showed clinical signs of atypical swimming behavior with increase respiration rate and stress, and dark color of fish body surface (data not shown).

To evaluate the histopathologic effects of formalin on gills, liver, skin, and eyeball under formalin bath for 10 min and 1, 2, 4, 8, 24, 48, and 96 h. Under 200 ppm formalin bath, a lot of mucus production on the tilapia gills. A large of gill epithelial cells shed and column cell degenerated. Under 50 ppm formalin bath for 10 min, gill damage and a lot of mucus production showed on the surface of tilapia gills (Figure 2A-C). A slight symptom of 50 ppm formalin-treated fish was found compared to the group with 200 ppm formalin bath. The respiration rate was slight increase and swimming behavior was normal under 50 ppm formalin bath. After 24 h formalin bath, a lot of mucus production was disappeared, however the degeneration of column cells was not return normality (Figure 2D-F). Additionally, no pathologic difference in liver, skin, and eyeball was revealed (data not shown). Under 48 and 96 h-formalin bath, the slight improvement was found in the damage tissues (Figure 2G-J).

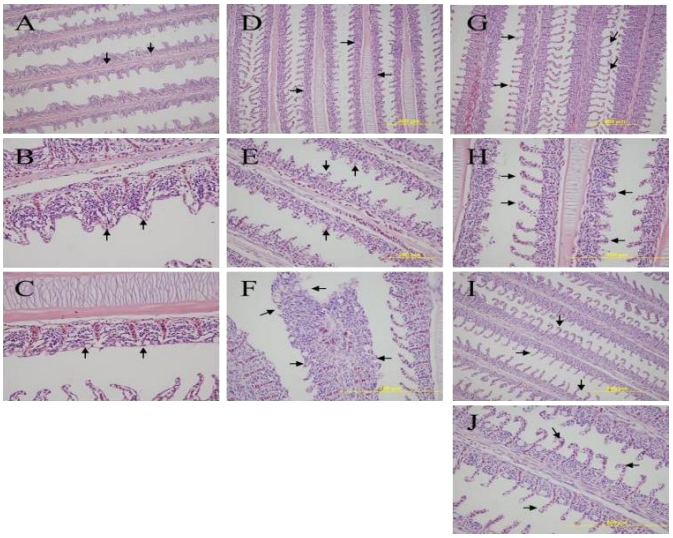

Figure 2: A lot of mucus on the tilapia gill following exposure to 50 ppm formalin 10 $min^{(A-C)}$, 24 $h^{(D-F)}$, 48 $h^{(G-H)}$, and 96 $h^{(I-J)}$. (A-C) A large number of gill filament epithelia shedding, congestion on the top of gill filaments, and degeneration of column cells were represented following exposure to 50 ppm formalin 10 min. (D-F) Reducing of mucus secretion, degeneration of column cells and rupture of gill lamellae, hypertrophy of cells on the top of gill filaments, and rupture of gill were represented following exposure to 50 ppm formalin 24 h. (G-J) Reducing of mucus secretion and the situation has gradually improved following exposure to 50 ppm formalin 48 h and 96 h. (bar = 200 nm).

7.2. Simultaneous Determination of Formalin by High Performance Liquid Chromatography with Ultraviolet Detection

The high performance liquid chromatography with ultraviolet detection analysis for the simultaneous determination of formalin was rapid with a high degree of reproducibility. The results showed an obvious peak (wavelength 360 nm) of 1 ppm formalin at 6.2 min under the ultraviolet detector (Figure 3). The standard curve was Y = 600801X - 95832 (X: concentration; Y: area), r^2 value of 0.9957 (Figure 4).

All HPLC chromatographs of formalin at various concentrations (0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 ppm) were presented that formalin peak occurred at 6.2 min (data not shown). According to these results, HPLC method with ultraviolet detection was able to successfully detect formalin. Additionally, formalin (0.2, 2, and 20 ppm) was used for detection inter-day and intra-day precision and accurate of the method. The coefficient of variation (C.V.) of intra-day precision and accurate was between 0.01% to 0.09% (the mean C.V. = 0.057%) (Table 1). The C.V. of inter-day precision and accurate was between 0.005% to 0.15% (the mean C.V. = 1.53%) (Table 1). The C.V. of inter-day and intra-day precision and accurate was lower than 10% of the suggestion of food and drug administration, USA. The precision and accurate is accepted in this study. Moreover, LOQ and LOD was respectively 5 (s/n≥10) and 0.1 (s/n≥3) ppb. The muscle or liver concentrations below this value were not used for the pharmacokinetic analysis.

The recoveries (%) of formalin in the spiked muscle and liver of tilapia were 104% and 100%, respectively (Table 2). HPLC chromatographs of formalin at 20 ppm in tilapia muscle and liver was presented that formalin peak occurred at 6.2 min (Figure5).

Respectively, the concentrations of formalin in muscle, liver, and cultured water following bath of 25 and 50 ppm formalin in the experimental tanks. The concentrations of formalin in muscle, liver, and cultured water at the various time points (1, 2, 4, 8, 24, 48, and 96 h) of formalin bath were

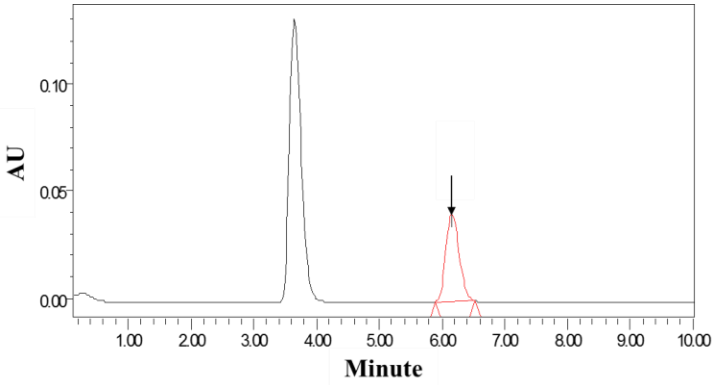

Figure 3: HPLC-UV chromatographs for 1 ppm formalin standard solution.

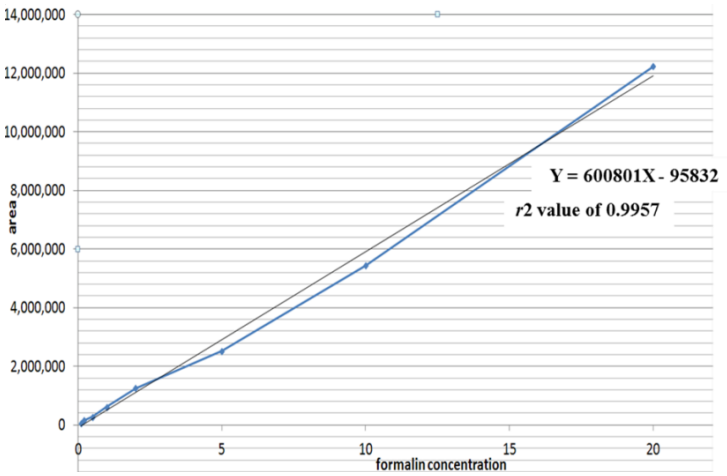

Figure 4: The standard curve of spiked formalin (0.1, 0.2, 0.5, 1, 2, 5, 10, 20 ppm) by HPLC with UV detection.

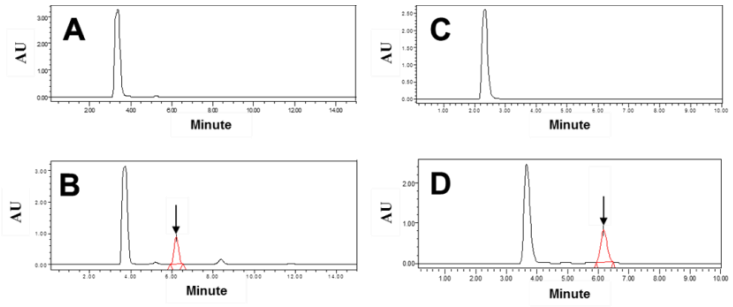

Figure 5: HPLC-UV chromatographs for formalin in the spiked muscle and liver of tilapia. (A) blank tilapia muscle, (B) tilapia muscle spiked with 20 ppm formalin, (C) blank tilapia liver, and (D) tilapia liver spiked with 20 ppm formalin. Arrow represented formalin appeared at 6.2 min.

shown in (Table 3 & 4). After 1 h-formalin (25 ppm) bath, formalin concentration in the muscle of tilapia was 9.45 ± 0.11 ppm, however, non-detection of formalin was presented in the cultured water and liver of tilapia. After 2 h-formalin (25 ppm) bath, formalin concentration in the muscle of tilapia was 2.7 ± 0.04 ppm. After 4 h-formalin (25 ppm) bath, formalin concentrations in cultured water, muscle, and liver were not detected (Table 3).

After 1 h-formalin (25 and 50 ppm) bath, formalin concentration in the cultured water was below LOD (0.1 ppb). After 1 and 2 h-formalin (50 ppm) bath, formalin concentration in cultured water of tilapia was 10.8 and 2.7 ppm, respectively. At 4 h of formalin (50 ppm) bath, formalin concentration in cultured water was below LOD (0.1 ppb). After 1 and 2 h-formalin (50 ppm) bath, formalin concentration in the liver of tilapia was both 0.16 ppm. At 4 h of formalin (50 ppm) bath, formalin concentrations in cultured water, muscle, and liver of tilapia were below LOD (0.1 ppb) (Table 4).

8. Discussion

During 1993-2000 years, tilapia culture has been one of the most important aquaculture sectors in Taiwan and the most of the products are export-

Table 1: Accuracy and precision for the determination of formalin (0.2, 2, and 20 µg/mL) by using high performance liquid chromatography with ultraviolet detection.

Day		Formalin (µg/mL)	Area (mean)	S.D.[a]	C. V. [b] (%)
1st	Intra-day	20	12,296,591	119,361	0.01
		2	1,314,460	111,909	0.09
		0.2	121,445	8,493	0.07
1st	Inter-day	20	12,182,776	588,221	0.005
		2	1,374,558	159,099	0.12
		0.2	113,214	13,445	0.12
2nd		20	12,119,629	717,583	0.06
		2	1,386,216	52,655	0.04
		0.2	121,124	17,864	0.15
3rd		20	12,199,666	288,650	0.02
		2	1,358,411	63,480	0.05
		0.2	118,594	7364	0.06

[a]Standard derviation; [b]Coefficient of variation

Table 2: Recoveries of 20 ppm formalin from spiked muscle and liver of tilapia (n = 3/group).

Tissue	Original concentration (ppm)	Spiked tissues (ppm)	Recovery (%)
Muscle	20	20.8 ± 0.05	104
Liver	20	20.1 ± 0.02	100

Table 3: Pharmacokinetics describing the disposition of 25 ppm formalin in the culture water, liver, and muscle of tilapia (n = 6/group).

	Mean ± SD[b] (ppm)		
Time (h)	H_2O	Liver	Muscle
1	URL [a]	URL [a]	9.45 ± 0.11
2	URL [a]	URL [a]	2.7 ± 0.04
4	URL [a]	URL [a]	URL [a]
8	URL [a]	URL [a]	URL [a]
24	URL [a]	URL [a]	URL [a]
48	URL [a]	URL [a]	URL [a]
96	URL [a]	URL [a]	URL [a]

[a]URL: Under detected limit; [b]Standard derviation

ed to the global markets [4]. Development of tilapia industry is an important issue in the wider context of Taiwan and global food supplies. Thus, the use of drugs in tilapia aquaculture became very importance. Formalin is wildly used in the sanitization and prevention of pathogen infection in veterinary medicine [4]. Due to its pathological and physiological effects were well-known, its use in animal husbandry has the potential to result in the presence of residues in tissues that could have potential health risks to humans[(4)], therefore, use of formalin must be careful.

Table 4: Pharmacokinetics describing the disposition of 50 ppm formalin in the culture water, liver, and muscle of tilapia (n = 6/group).

	Mean ± SD[b] (ppm)		
Time (h)	H_2O	Liver	Muscle
1	URL [a]	0.16	10.8
2	URL [a]	0.16	2.7
4	URL [a]	URL [a]	URL [a]
8	URL [a]	URL [a]	URL [a]
24	URL [a]	URL [a]	URL [a]
48	URL [a]	URL [a]	URL [a]
96	URL [a]	URL [a]	URL [a]

[a]URL: Under detected limit; [b]Standard derviation

It is an important issue whether formalin retained in fish tissues via gills and skin following exposure to formalin. Previously, formalin residues, 5.75 g/g in sliver barb (*Puntius gonionotus Bleeker*) muscle and 14.03 g/g in walking catfish (*Clarias batrachus* L.) muscle under 50 ppm formalin bath for 24 h, was demonstrated [13]. On the other fish species, no significant difference in formalin residues in the muscle of rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), largemouth bass (*Micropterus salmoides*), and channel catfish (*Ictalurus punctatus)* following exposure to formalin for respectively 1 and 3 h compared to the control group[(12)]. The similar results were as well as in olive flounder (*Paralichthys olivaceus*), Nile tilapia (*Tilapia niloticus*), and blue shrimp (*Penaeus stylirostris*) [14-16]. In this study, formalin residues in the tilapia muscle and liver, and cultured water were below LOD (0.1 ppb) within 4 h-formalin (25 and 50 ppm) exposure. Additionally, formalin residue in the cultured water was disappeared within 1 h-formalin (25 and 50 ppm) exposure. According to these data for formalin residue in fish, fish species may be a reason for the degree of formalin residue in fish muscle.

Fish species may be a reason to affect formalin tolerance. Cruz and Pitogo [17] reported that the 24-, 48-, 72-, and 96-h LC_{50} were 322, 260, 241, and 232 ppm formalin, respectively in milkfish (*Chanos chanos*). Fajer-Ávila et al. [18] presented the formalin LC_{50} for the bullseye puffer fish (*Sphoeroides annulatus*) ranged from 1095 mg/L at 30 min and 972 mg/L at 60 min to 79 mg/L at 72 h. In this study, the formalin 48 h-LC_{50} and 96 h-LC_{50} was 179 and 177.2 ppm, respectively. Additionally, other factors like gill function, acid-base balance, pituitary activation occurring, water temperature, pH value, fish health, and culture filtration system also affected formalin toxicity [19-21].

Tilapia culture and export in Taiwan were very important industries. Formalin is an effective chemotherapeutant which is used to control certain ectoparasitic and bacterial infections in fish disease therapies. Unfortunately, it is toxic to fish and its pathological and physiological effects have

been also reported. Due to formalin is an effective sanitizer and it was widely used in veterinary, the drug residues in tilapia tissues was an important issues [12]. Although the level of formalin residue in fish tissues is not detected based on the Standards of Veterinary Drug Residues in Aquatic Animal Drugs Use in Taiwan, however, understanding the residue and pharmacokinetics of formalin in fish tissues is necessary when food and safety problems occurred in the future in Taiwan. In this study, after 1 h-formalin (25 ppm) exposure, formalin was not detect in liver and cultured water of tilapia. It was only detected the concentration of 9.45 ± 0.11 ppm in muscle. After 4 h-formalin (50 ppm) exposure, formalin concentrations in the muscle, liver, and cultured water were below LOD (0.1 ppb). Thus, the elimination rate of formalin in tilapia muscle and liver, and cultured water was rapidly. Within 1 h-formalin (25 and 50 ppm) exposure, the formalin concentration in cultured water was below LOD and within 4 h-formalin (25 and 50 ppm) exposure, the formalin concentrations in tilapia muscle and liver were below LOD.

For a long time, formalin was used for the disease prevention and therapies in ornamental fish in Taiwan and in salmon and trout in USA. Many methods have been described for the determination of formalin worldwide. The method, 2,4-dinitrophenylhydrazin derivatization with solid/liquid phase extraction and HPLC, was the major method for formalin detection in many countries [12]. In Taiwan, formalin detection was performed by using HPLC with ultraviolet detection in water (NIEA W782.5OB) and 4-amino-3-hydrazino-5-merca- pto-1,2,4-triazol colorimetry in air (NIEA A724.72). Unfortunately, the detection method for formalin residue in animal tissues is lack in Taiwan until now. Thus, the development of a simple, rapid, and reliable technique to detect the pharmacokinetics and the residual of formalin in animal tissues is need. In this study, these data presented that formalin retained time of 6.2 min, formalin LOQ and LOD of 5 and 0.1 ppb, and the recoveries (%) of formalin in the spiked muscle and liver of tilapia were 104% and 100%, respectively. Thus, using of HPLC with ultraviolet detection and its procedures can produce satisfactory results for detecting formalin pharmacokinetics in the muscle and liver of tilapia.

Based on the tilapia is one of the most important fish species and formalin was a wildly used sanitizer in fish farms. A portion of administered pharmaceuticals remains in the animal's body, but a significant fraction is discharged into the environment by excretion. The ensuing risks of pharmaceutical residues reaching edible products and the potential health hazards associated to their consumption have become a public safety issue [5]. Therefore, the knowledge of pharmacokinetic studies of formalin in tilapia will be very important. Taken together, the detection of formalin concentrations in muscle and liver was following exposure to formalin (25 ppm) at 2 h in muscle and at 1 h in liver were both below LOD (0.1 ppb). Under 50 ppm formalin exposures for 4 h, both muscle and liver with formalin residues were below LOD (0.1 ppb). Under 25 and 50 ppm formalin exposure for 1 h, a formalin residue in cultured water was below LOD (0.1 ppb). Thus, the elimination rate of formalin in fish tissues and cultured water was rapidly, therefore, 25 and 50 ppm formalin are safety in tilapia and its drug residue should be notworry.

9. Conclusion

A method for the determination of formalin in hybrid tilapia, *Oreochromis hybrids* was developed. Precipitation/centrifugation/filtration of on a mixture of animal tissue with acetonitrile-water and subsequent continuous solid-phase extraction provide a reliable procedure for removing co-extracting interferences (proteins and lipids, mainly) from complex matrices and facilitate preconcentration of the pharmaceuticals. The method is quite sensitive (LOD: 1 ppb; LOQ: 5 ppb), accurate, and precise. The pharmacokinetics of formalin were studied in tilapia following exposure indicated the elimination rate of formalin in tilapia or cultured water was very rapidly. To the best of our knowledge, this is the first report of pharmacokinetic studies was done in tilapia in Taiwan. The pharmacokinetic information derived for tilapia will be used to design rational dosing regimens and also assist the Taiwan government to formulate the laws and regulations for formalin residue in tilapia in the future. The study will be showed to people the drug residue problem of formalin in fish is not so terrible. The main damage in fish was focus at gill not drug residue. Therefore, formalin is the most economical and effective drugs for parasitic aquatic parasite treatment nowadays. Additionally, this information will be provided drug selection and dosage reference to breeders and veterinarians and the deeper protection on aquatic agricultural productions for consumers.

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