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Research Article

Acute Toxicity and Drug Residue Analysis of Formalin in Two Cold Water Fishes: Cultured Hybrid Bester Sturgeon (*Huso huso* × *Acipenser ruthenus*) and Rainbow Trout (*Oncorhynchus mykiss*)

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1. Abstract

Formalin is approved for use in aquaculture for controlling ectoparasites and aquatic fungi. The safety of using formalin-treated aquatic animals for food has been widely discussed in the aquaculture industry. The aim of this study was to determine the residual concentrations of formalin in the muscle and liver of the cultured Bester sturgeon and rainbow trout by HPLC-UV analysis. Results showed that the median lethal concentration was progressively reduced as the duration of exposure increased. The LC_{50} values at 48 h in sturgeon and rainbow trout were 216 and 211 ppm, respectively. Under 50 ppm formalin treatment, gill lamellar epithelium separation, hypertrophy, and necrosis were observed via histopathological examination. However, the gill lesions had recovered after 96-h formalin exposure. Meanwhile, the time to undetectable residue levels of formalin in the liver and muscle of sturgeon after 25 ppm formalin treatment was 48 and 96 h, respectively, and after 50 ppm formalin treatment, it was below 1 h. In rainbow trout, the time to undetectable residue levels of formalin in the liver and muscle after 25 ppm formalin treatment was, respectively, 96 and 240 h and after 50 ppm formalin treatment they were 8 and 24 h, respectively.

2. Keywords

Cold water fish; Formalin; High performance liquid chromatography; Rainbow trout; Sturgeon

3. Abbreviations: CV: Coefficient of Variation; FDA: Food and Drug Administration; HPLC: High Performance Liquid Chromatography; IACUC: Institutional Animal Care and Utilization Committee; LC_{50}: Median Lethal Concentration; SD: Standard Deviation; SPE: Solid Phase Extraction; URL: Under Detected Limit; UV: Ultraviolet

4. Introduction

Formalin has been used to control protozoan parasites in fish and crustaceans. It is used in industrial applications, as a disinfectant and biocide, as a tissue fixative and embalming agent, in drug testing, and in a variety of hobbies [1-3]. Formaldehyde is highly toxic to all animals, regardless of intake method. Ingestion of as little as 30 mL (1 oz.) of a solution containing 37% formaldehyde (formalin) has been reported to cause death in an adult human. An aqueous 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 concern with respect to human health [4].

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Sturgeons and rainbow trout are cold water fishes and commercially important fish species worldwide. Thus, sturgeon and rainbow trout aquaculture and development are important issues in the wider context of global food supply [5-7]. The high population of animals grown in agricultural facilities and fish hatcheries can increase the potential for disease outbreaks. This raises the need for medications therapeutically to treat existing infections, or prophylactically, to minimize the impact of an outbreak spreading across an animal population. Bacterial diseases remain a major problem in aquaculture and account for significant losses of fish. Antimicrobial therapy presently has an important role to play in aquaculture [8]. The use of antibacterial compounds in fisheries is a highly regulated issue because of the possibility of tissue deposition and development of bacterial resistance [9].

Sanitizers are commonly used in aquaculture for disease prevention. Among these, formaldehyde is effective against pathogens and has been widely used in fish culture [9-11]. However, only a handful of known concentrations of formaldehyde are available to be used effectively and without damage to fish. Thus, pharmacokinetic investigations are important to define appropriate dosage regimens for formalin. In tilapia, formalin has been demonstrated to have immunosuppressive effects and reproductive toxicity [4,12]. Reproductive toxicity has also been shown in humans, mice, and rats [13-15]. In order to adequately protect consumers and secure dairy and aquacultural production, 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, although there is no appropriate concentration to be used effectively and without damage to tilapia [12]. 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 the Food and Drug Administration (FDA) in the USA as parasiticides for use on fish. Thus, a proper withdrawal period for formalin is needed after drug administration to ensure that drug residues in edible tissues are below established tolerance levels.

At present, drug residues leading to drug resistance in pathogens have 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 consumer's health [15]. Additionally, pharmacokinetic profiles of formalin had not been investigated in sturgeon and rainbow trout, which are commercially important fish species in Taiwan and worldwide. Since pharmacokinetic studies and data on the withdrawal period of formalin in sturgeon and rainbow trout are lacking, development of a simple, rapid, and reliable technique to detect the pharmacokinetics of formalin will help researchers to understand formalin metabolism in sturgeon and rainbow trout. Thus, the objectives of this study were to determine the pharmacokinetic parameters of formalin in sturgeon and rainbow trout following single-dose bath administration. Based on the pharmacokinetics of formalin in sturgeon and rainbow trout, this test will be applied to the prevention of infectious diseases on site. The trial was the first study to investigate the pharmacokinetics of formalin before and after various periods of administration in farmed sturgeon and rainbow trout using high performance liquid chromatography (HPLC) with ultraviolet (UV) detection. These data will be a reference basis for the withdrawal period of formalin in the sturgeon and rainbow trout industries in Taiwan and worldwide.

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 double deionized water at the stock concentration of 1,000 ppm and stocked at -20°C. The stock solution of formalin was diluted with double deionized water to 0.1, 0.2, 0.5, 1, 2, 5, 10, and 20 ppm, before beginning the experiments. Other analytical grade chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

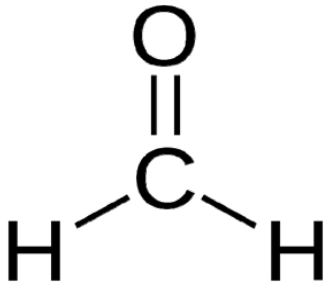

Figure 1: Chemical structure of formaldehyde.

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 5C18-MS column (5 μm, 4.6 × 150 mm i.d.; Nacalai, Kyoto, Japan). The UV detector (model 2475, Waters) with auto-change wavelength and the wavelength was set to 360 nm in this study. Analysis of HPLC data was performed using the 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 the IACUC and the United States National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Healthy sturgeon, a cultured *Huso huso* × *Acipenser ruthenus* hybrid, (body weight: 130 ± 10 g) and healthy rainbow trout, *Oncorhynchus mykiss* (body weight: 100 ± 10 g) were obtained from the Fisheries Research Institute, COA (Changhua county, Taiwan) and a cultural fish farm (Syuejia District, Tainan City, Taiwan). These fish were routinely examined by the aquatic veterinarian of the Taiwanese government to determine whether pathogenic bacterial or parasitic infections were found in these fish. The fish were housed in a 474 L experimental tank (length: 120 cm × width: 60 cm × depth: 70 cm) with running and UV filtered re-circulating aerated fresh water (Temperature: 19°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 sturgeon and rainbow trout pellets purchased from the feed factory (FWUSOW industry Co., Ltd, Tainan, Taiwan) and kept on a 12 h light/dark cycle. The composition of the sturgeon basal diet included more than 45% crude protein, more than 4% crude fat, more than 16% ash content, less than 3% crude fiber, less than 11% moisture, and less than 3% insoluble matter in hydrochloric acid; the composition of the rainbow trout basal diet included more than 43% crude protein, more than 3% crude fat, less than 15% ash content, less than 3% crude fiber, less than 11% moisture, and less than 2% insoluble matter in hydrochloric acid. Fish were humanely euthanized with tricaine methanesulfonate (MS222; Sigma-Aldrich) for sample collection. The muscle and liver of sturgeon and rainbow trout were obtained from ten untreated fishes and no formalin residue was identified in these samples.

5.4. Experimental Design and Sample Collection

First, the organs were collected, histopathology examined, and mortalities recorded from ten small-size fishes per group after bathing with the various concentrations of formalin (150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 300, 400, and 500 ppm) for 2, 4, 6, 24, 48, 72, and 96 h. Additionally, 30 large-size fishes/group bathed with 25 or 50 ppm formalin were also collected. The age- and size-matched control fish bathed with fresh water without formalin. After bathing with or without formalin for 1, 2, 4, 8, 24, 48, 72, and 96 h, six fishes/group at each experimentalpoint were caught randomly for quantification of formalin residues in the muscle and liver.

5.5. Histopathologic Examination

Histopathologic examination was carried out as described previously [16-17]. After bathing in the various concentrations of formalin, fish were humanely euthanized with tricaine methanesulfonate (MS222; Sigma-Aldrich). Gill, liver, skin, muscle, and eyeball samples were aseptically collected and submitted for histopathologic examination. Tissues were dehydrated by serial ethanol and xylene stages for automated processing into wax blocks and 5 μm sections were examined. All slides were stained with hematoxylin and eosin (HE) (BBC Biochemical, USA) and observed microscopically.

5.6. Sample Pretreatment and Derivatization

Sample pretreatment and derivatization were carried out as described previously [16-17] and modified. Respectively, 2 g of muscle and 2 g of liver tissues from Bester sturgeon and rainbow trout were immediately well mixed with 10 mL extraction solution [a mixture of 1.0 N NaOH (64.3 mL) and glacial acetic acid (5.7 mL) in 930 mL double deionized water; pH 4.93 ± 0.02]. Subsequently, each mixture was homogenized using a homogenizer (OMNI Macro ES, NY, NY, USA) at 10,000 rpm for 1.5 min and ultra-sonication for 10 min. Finally, the samples were centrifuged at 6,000 rpm for 10 min to collect the supernatants and sediments. The sediments were immediately well mixed with 10 mL extraction solution and the above procedures repeated to collect the supernatants again. These supernatants from the muscle and liver samples were evenly mixed with 6 mL 2, 4-dinitrophenylhydrazine (3 g/L) and 4 mL acetate buffered solution in a water bath (Bransonic 8210, Branson Inc., Danbury, CT, USA) at 60°C and 60 rpm for 20 min. Finally, samples were allowed to cool to 20°C in cold water and the cool mixture was evenly mixed with 10 mL saturated NaCl(aq) and filtered through a 0.45 μm PVDF syringe filter (Acrodisc, Pall Inc., NY, NY, USA) before HPLC analysis. Additionally, culture water was also collected at the sample collection time points to analyze 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 were carried out according to previously described procedures [18].

5.8. Derivatization

Derivatization was carried out as described previously [16-17] with some modifications. The extracted and cleaned up sample (0.1 mL) was evenly mixed with 20% TCA (100 μL) for 5 sec, then immediately well-mixed with 200 μL 7% formaldehyde (Merk, Darmstadt, Germany). Later, DDW was added to give

a total volume of 1 mL and the mixture incubated at 100°C for 30 min. Finally, the sample was cooled to 20°C in cold water and filtered through a 0.2 μm PVDF syringe filter (Acrodisc, Pall Inc., NY, NY, USA) before HPLC analysis.

5.9. Method Validation

Method validation was carried out as described previously [16-17] with some modifications. Linearity was assayed according to the slopes, intercepts, and correlation coefficients of the calibration curves calculated by linear regression analysis. The recovery was determined by repetitively analyzing blank muscle (2 g) and liver (2 g) supplemented with known amounts of formalin (20 ppm) or un-supplemented. After derivatization, formalin recovery was performed using HPLC with ultraviolet detection. Recovery was calculates according to the following formula: Recovery = (the eluted formalin concentration in samples / the accurate adjunction of formalin concentration in samples) × 100%.

5.10. Limit of Detection and Limit of Quantification

Limit of detection and limit of quantification were estimated as described previously [16-17]. 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

Stability of formalin in extracts was determined as described previously [16-17]. 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

Determination of formalin was carried out as described previously [16-17] with some modifications. Samples (20 μL) were analyzed on a Waters quaternary solvent delivery system (model 600e) (Waters, Milford, MA, USA). Separation was achieved on a Cosmosil $5C_{18}$-MS column (5 μm, 4.6 × 150 mm i.d.; Nacalai, Kyoto, Japan), which was 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-1 for 1 h in the 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 to 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. An ultraviolet detector (model 2475, Waters) was used with the wavelength of 360 nm. Analysis of HPLC data was performed using the Millennium 32 software (Version 4.0, Waters).

5.13. Assay Validation

The intra-assay and inter-assay validation of formalin was carried out according to previously described procedures [18]. The concentration of formalin in samples was determined using the linear regression line (unweighted) of the concentration standard versus peak area. 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}) according to the following formula: bias (%) = $[(C_{obs} - C_{nom}) / (C_{nom})] \times 100$.

The relative 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 previously [18]. Three concentrations of formalin (0.2, 2, and 20 ppm) were analyzed three times each 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 the 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 Bester Sturgeon and Rainbow Trout

The acute toxicity assay was performed in Bester sturgeon, *Huso huso* × *Acipenser ruthenus*, and rainbow trout at several time points (2, 4, 6, 24, 48, 72, or 96 h) of formalin (150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 300, 400, or 500 ppm) exposure (bath treatment) to evaluate clinical behavior and mortality of Bester sturgeon and rainbow trout. Each group (n = 10 fishes) was bathed with formalin for a range of time points to calculate the 50% lethal dose (LD_{50}) of formalin and the 95% confidence interval following a 96 h formalin bath. In groups bathed with 200 to 500 ppm formalin, all 20 fish died. However, in the group bathed with 150 ppm formalin, all 20 fish survived. The 48 h LD_{50} values in sturgeon and rainbow trout were, respectively, 216 and 211 ppm and the 95% confidence intervals were 2.335 and 2.326, respectively (**Table 1**). Clinical examination of the Bester sturgeon and rainbow trout after a formalin (230 to 300 ppm) bath

of 1-2 h showed clinical signs of atypical swimming behavior with increased respiration rate and stress and dark color of the fish body surface (data not shown).

Table 1: Acute toxicity test of formalin in sturgeon and rainbow trout between 2 h- to 48 h-formalin exposure. Data shown are the 48 h LD_{50}values in sturgeon and rainbow trout were, respectively, 216 and 211 ppm and the 95% confidence intervals were 2.335 and 2.326, respectively

formalin (ppm)	sturgeon					rainbow trout				
	2 h	4 h	6 h	24 h	48 h	2 h	4 h	6 h	24 h	48 h
160	10/10	10/10	10/10	10/10	9/10	10/10	10/10	10/10	9/10	9/10
170	10/10	10/10	10/10	8/10	8/10	10/10	10/10	9/10	9/10	8/10
180	10/10	10/10	10/10	9/10	9/10	10/10	10/10	9/10	8/10	6/10
190	9/10	9/10	9/10	9/10	9/10	10/10	10/10	10/10	8/10	6/10
200	10/10	8/10	7/10	7/10	6/10	10/10	10/10	10/10	9/10	7/10
210	9/10	7/10	7/10	7/10	6/10	10/10	10/10	8/10	8/10	6/10
220	10/10	8/10	6/10	6/10	4/10	10/10	9/10	6/10	6/10	5/10
230	8/10	5/10	5/10	5/10	5/10	8/10	8/10	7/10	5/10	5/10
240	7/10	6/10	4/10	4/10	4/10	7/10	6/10	6/10	5/10	4/10
250	7/10	5/10	3/10	2/10	2/10	6/10	6/10	5/10	3/10	2/10
260	5/10	4/10	3/10	1/10	0/10	4/10	4/10	2/10	2/10	0/10

7.2. Clinical Fish Behavior and Histopathological Effects after Formalin administration

The histopathologic effects of formalin on the gills, liver, skin, and eyeball after formalin bath for 10 min and 1, 2, 4, 8, 24, 48, or 96 h were examined. Initially, in the 200 ppm formalin bath, a large proliferation of mucus can be observed. The epithelial cells and columnar cells of the gill were defaced and denatured after 200 ppm formalin bath. Following the 200 ppm formalin bath, normal breathing in the fish was also affected. In the first hour after 200 ppm formalin bath, the fish began to exhibit a critical state; the body surface became black and the respiratory rate increased. After exposure to the 200 ppm formalin bath for 2 h, the fish began to float on the water surface or stay in the corner of the experimental tanks. Conversely, symptoms were much less pronounced in the fish bathed with 50 ppm formalin, compared to those in the group bathed with 200 ppm formalin. The respiration rate slightly increased and swimming behavior was normal in the 50 ppm formalin bath. Mucus began to be produced on the gill tissues exposed to 50 ppm formalin for 1 h. Wide spread epithelial shedding on gill filaments, engorgement of top gill filaments, and degeneration of column cells were also observed in sturgeon and rainbow trout (**Figures 2A** and **3A**).

In sturgeon bathed with 50 ppm formalin for 24 h (**Figure 2B**) and rainbow trout bathed with 50 ppm formalin for 12 h (**Figure 3B**), mucus secretion was reduced and column cell degeneration, rupture of the gill lamellae, hypertrophy of apical cells, and the rupture of gills filaments were observed.

In sturgeon bathed with 50 ppm formalin for 48 h (**Figure 2C**) and rainbow trout bathed with 50 ppm formalin for 24 h (**Figure 3C**), the gill filaments appeared fused. In sturgeon and rainbow trout bathed with 50 ppm formalin for 96 h, the extent of gill damage caused by formalin was reduced **(Figures 2D** and **3D**). In sturgeon and rainbow trout bathed with 50 ppm formalin for 168 h, mucus secretion was reduced and symptoms had gradually improved (**Figures 2E** and **3E**). Additionally, no pathologic differences in the liver, skin, or eyeball were observed (data not shown).

7.3. Retain time of Formalin Under in the Different Mobile Phases Composited with the Double Distilled Water/Acetonitrile Effect

To investigate whether different mobile phases composited with double distilled water/acetonitrile affect the retain time of formalin, we used mixtures of double distilled water/acetonitrile in various proportions (50/50, 60/40, 70/30, and 80/20). The retain time of formalin was 2.997, 3.447, 4.845, and 6.156 min in the different mobile phases (**Figure 4**).

7.4. The degree of Reproducibility of Formalin Detection

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 6.156 min under the ultraviolet detector (**Figure 5**). The standard curve was Y = 60080X - 95832 (X: concentration; Y: area) with r^2 value of 0.9957 (**Figure 6**).

7.5. Inter-day and Intra-day Precision and Accuracy of Formalin Detection

All HPLC chromatographs of formalin at various concentrations (0.1, 0.2, 0.5, 1, 2, 5, and 10 ppm) showed that the formalin peak appeared at approximately 6.2 min (data not shown). These results demonstrated that the HPLC method with ultraviolet detection was able to successfully detect formalin. Additionally, formalin (0.2, 2, and 20 ppm) was used to assess the inter- and intra-day precision and accuracy of the method. The coefficient of variation (CV) of intra-day precision and accuracy was between 0.01% and 0.09% (mean CV = 0.057%) (**Table 2**). The CV of inter-day precision and accuracy was between 0.005% and 0.15% (mean CV = 0.069%) (**Table 2**). The CV of inter- and intra-day precision and

accuracy was lower than 10% of that suggested by the FDA. The precision and accuracy were acceptable in this study. Moreover, the LOQ and LOD were 5 (s/n≥ 10) and 0.1 (s/n ≥ 3) ppb (ng/mL), respectively. Any muscle or liver concentrations below this value were not used in the pharmacokinetic analysis.

7.6. Recoveries (%) of Formalin in the spikedMuscle and Liver of Sturgeon and Rainbow Rrout

The recoveries (%) of formalin in the spiked muscle and liver of sturgeon were 94% and 95%, respectively (**Table 3**). The recoveries (%) of formalin in the spiked muscle and liver of rainbow trout were 96.5% and 97%, respectively (**Table 3**). According to the HPLC-UV chromatograms of 20 ppm formalin evenly mixed with the blank muscle and liver of sturgeon and rainbow trout, the peak of formalin appeared at approximately 6.2 min (**Figure 7**). Formalin approached 10.506 min in the HPLC-UV chromatographs for the muscle tissues of sturgeon and rainbow trout spiked with 20 ppm formalin (**Figure 8**).

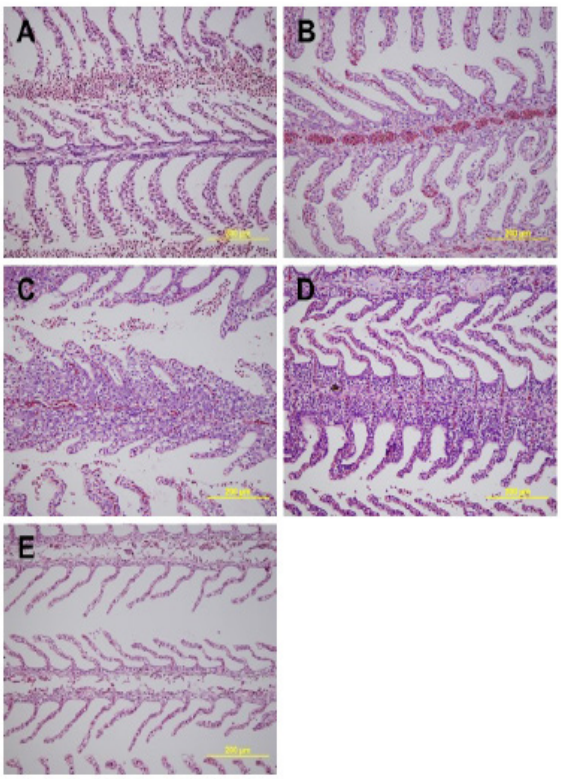

Figure 2: Histopathological effects on the gill of sturgeon after 50 ppm formalin administration. (A) Mucus production began on gill tissue exposed to 50 ppm formalin for 1 h. A large number of gill filaments showed epithelial shedding, engorgement on apical surfaces, and degeneration of column cells. (B) After bathing in 50 ppm formalin for 24 h, mucus secretion was reduced and column cells degeneration, gill lamellae rupture, cell hypertrophy on gill filaments, and the rupture were observed. (C) After bathing in 50 ppm formalin for 48 h, gill filaments showed signs of fusion. (D) Following a 50 ppm formalin bath for 96 h, symptoms of gill damage situation caused by formalin were reduced. (E) After a 50 ppm formalin bath for 168 h, mucus secretion reduced and symptoms had gradually improved.

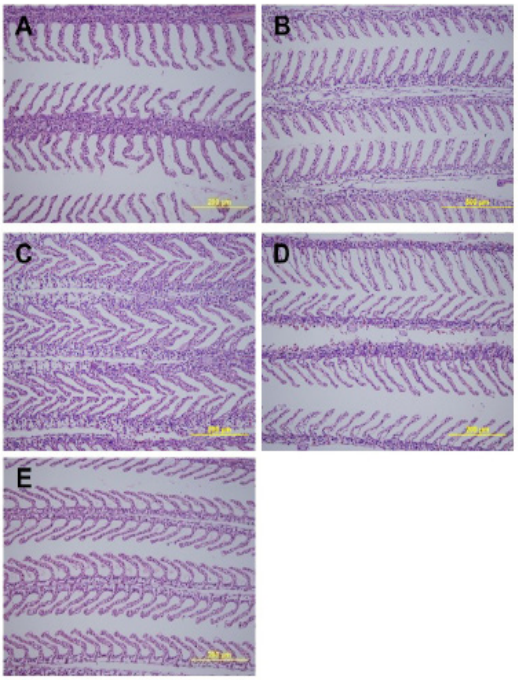

Figure 3: The histopathologic effects on gill tissues of rainbow trout after administration of 50 ppm formalin (A) Mucus began to be produced on gill tissue exposed to 50 ppm formalin for 1 h. A large number of gill filaments showed epithelial shedding; engorgement of the top of gill filaments and the degeneration of column cells were also observed. (B) After a 50 ppm formalin bath for 12 h, mucus secretion was reduced and the degeneration of column cells, the rupture of gill lamellae, and hypertrophy of cells on top gill filaments were observed. (C) Following a 50 ppm formalin bath for 24 h, gill filament showed signs of fusion. (D) After bathing in 50 ppm formalin for 96 h, symptoms of gill damage caused by formalin were reduced. (E) After 50 ppm formalin bath for 168 h, the mucus secretion reduced and symptoms had gradually improved.

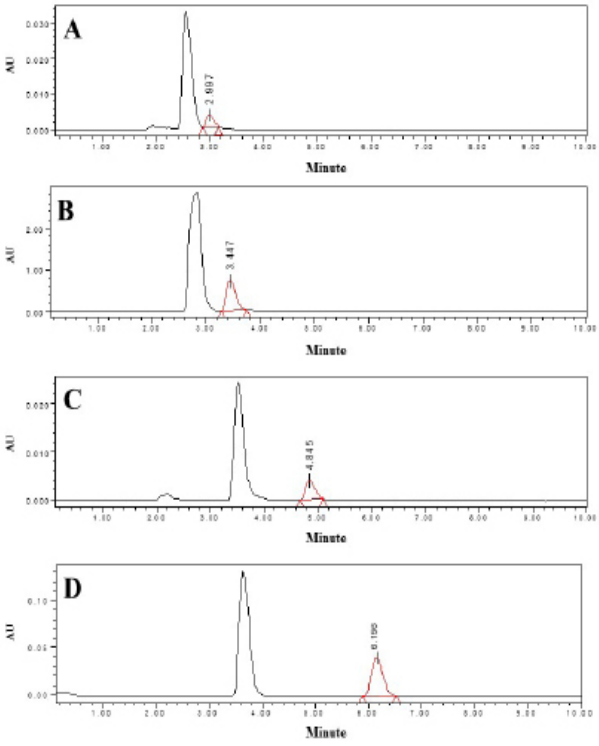

Figure 4: HPLC-UV chromatographs for 2 ppm formalin standard solution with different mobile phases composed of double distilled water and acetonitrile (A) 50/50 (B) 60/40 (C) 70/30 and (D) 80/20. Formalin respectively appeared at 2.997, 3.447, 4.845, and 6.156 min in the different mobile phases.

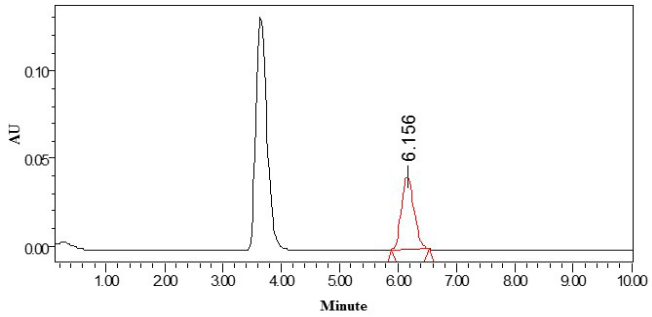

Figure 5: HPLC-UV chromatographs for 1 ppm formalin standard solution. Formalin appeared at 6.156 min.

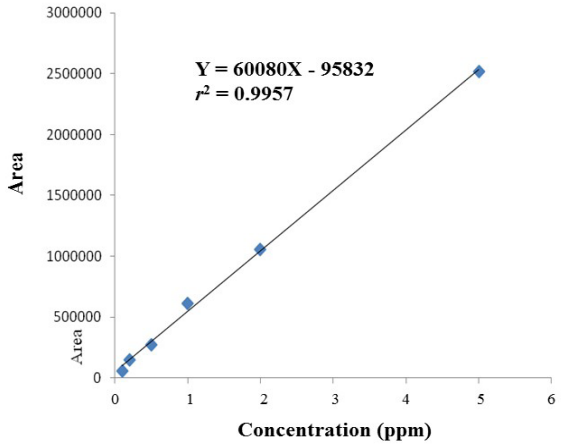

Figure 6: The standard curve of spiked formalin (0.1, 0.2, 0.5, 1, 2, 5, and 10 ppm) by HPLC with UV detection.

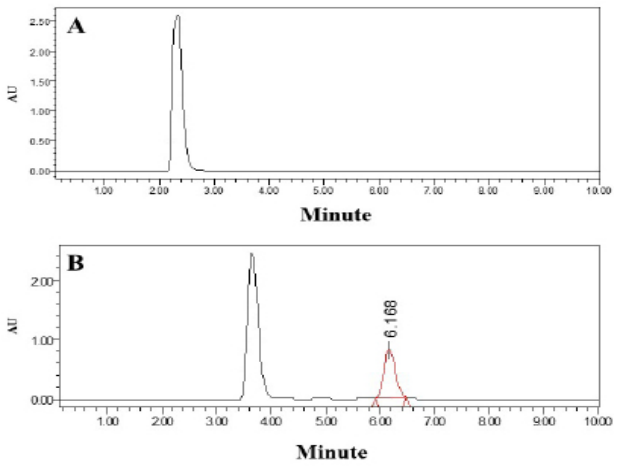

Figure 7: HPLC-UV chromatographs of the formalin standard. (A) blank and (B) 20 ppm formalin standard. Formalin appeared at 6.168 min.

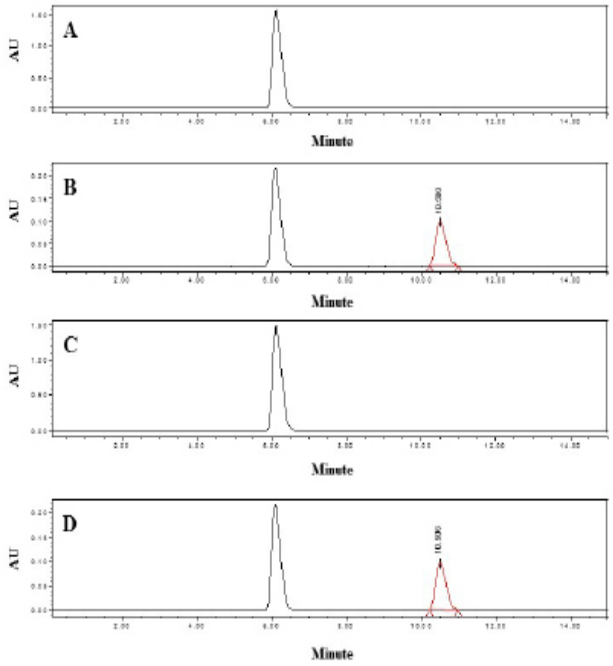

Figure 8: HPLC-UV chromatographs for formalin in the spiked muscle of sturgeon and rainbow trout. (A) blank sturgeon muscle (B) sturgeon muscle spiked with 20 ppm formalin (C) blank rainbow trout muscle, and (D) rainbow trout muscle spiked with 20 ppm formalin. Formalin appeared at 10.506 min.

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

Day	Formalin (ppm)	Area (mean)	SD	CV(%)
1st Intra-Day	20	71,216,545	619,361	0.01
	2	5,582,725	211,909	0.04
	0.2	503,416	35,239	0.07
1st Inter-Day	20	70,358,648	568,474	0.01
	2	5,589,143	286,451	0.05
	0.2	498,245	45,486	0.09
2nd Inter-Day	20	70,435,577	663,842	0.01
	2	5,981,246	246,853	0.04
	0.2	557,816	38,498	0.07
3rd Inter-Day	20	70,325,816	1,224,485	0.02
	2	5,615,849	265,487	0.04
	0.2	532,648	35,487	0.06

SD: Standard deviation; **CV:** Coefficient of variation

Table 3: Recoveries of 20 ppm formalin from spiked muscle and liver of sturgeon and rainbow trout (n = 3 per group).

Species	Tissues	Original concentration (ppm)	Spiked tissues (ppm)	Recovery (%)
Sturgeon	Muscle	20	18.9 ± 0.7	94
	Liver	20	19.1 ± 0.6	95
Rainbow trout	Muscle	20	19.3 ± 0.8	96.5
	Liver	20	19.4 ± 0.6	97

Table 4: Pharmacokinetics describing the disposition of 25 and 50 ppm formalin in the culture water, liver, and muscle of sturgeon (n = 6 per group).

	25 ppm formalin			50 ppm formalin		
Time (h)	H_2O	Liver	Muscle	H_2O	Liver	Muscle
1	7.1 ± 1.1	1.4 ± 0.2	URL	9.94 ± 1.13	1.67 ± 0.24	URL
2	5.51 ± 0.8	1.24 ± 0.36	URL	9.01 ± 0.88	1.43 ± 0.15	URL
4	5.08 ± 0.4	1.05 ± 0.08	URL	6.7 ± 1.4	1.27 ± 0.2	URL
8	2.35 ± 1.4	0.57 ± 0.05	URL	4.43 ± 1.22	1.05 ± 0.11	URL
24	0.7 ± 0.05	0.46 ± 0.12	URL	3.1 ± 0.76	0.9 ± 0.07	URL
48	URL	URL	URL	1.7 ± 0.55	0.43 ± 0.04	URL
96	URL	URL	URL	URL	URL	URL

URL: Under detected limit

Table 5: Pharmacokinetics describing the disposition of 25 and 50 ppm formalin in the culture water, liver, and muscle of rainbow trout (n = 6 per group).

	25 ppm formalin			50 ppm formalin		
Time (h)	H_2O	Liver	Muscle	H_2O	Liver	Muscle
1	8.66 ± 0.17	4.21 ± 0.13	2.64 ± 0.66	13.2 ± 2.13	2.17 ± 0.12	1.23 ± 0.24
2	6.93 ± 0.12	5.52 ± 0.14	4.4 ± 1.15	11.9 ± 2.05	2.76 ± 0.78	2.81 ± 0.11
4	4.87 ± 1.04	7.91 ± 0.8	1.19 ± 0.08	6.7 ± 1.88	3.54 ± 1.4	0.66 ± 0.04
8	2.3 ± 0.04	8.67 ± 1.52	URL	2.7 ± 1.45	3.96 ± 0.76	0.39 ± 0.07
24	1.06 ± 0.28	7.18 ± 2.03	URL	1.4 ± 0.04	4.07 ± 0.93	URL
48	URL	4.59 ± 0.14	URL	1.1 ± 0.03	3.66 ± 1.06	URL
96	URL	URL	URL	URL	2.81 ± 0.75	URL
168	URL	URL	URL	URL	1.9 ± 0.45	URL
240	URL	URL	URL	URL	URL	URL

URL: Under detected limit

7.7. Concentrations of Formalin in Muscle, Liver, and Culture Water

The concentrations of formalin in muscle, liver, and culture water at the various time points (1, 2, 4, 8, 24, 48, and 96 h) of formalin bath for sturgeon and rainbow trout are shown in (Table 4 and Table 5). After sturgeons were bathed for 1 h in formalin (25 ppm) bath, formalin concentrations in liver and culture water were 1.4 ± 0.2 and 7.1 ± 1.1 ppm, respectively. However, formalin was not detected in the muscle of sturgeon at any time point of formalin (25 or 50 ppm) exposure. Formalin concentrations in culture water and liver gradually decreased with time of formalin exposure. Formalin concentrations were under the limit of detection in culture water and the liver of sturgeon after 48 h formalin exposure (Table 4). Under 50 ppm formalin exposure in sturgeon, formalin concentrations in culture water and liver tissue gradually decreased with increased time of formalin exposure. Formalin concentrations were under the limit of in culture water and liver tissues of sturgeon after 96 h formalin exposure (**Table 4**).

After 1 h formalin (25 ppm) bath in rainbow trout, formalin concentrations in cultural water, liver, and muscle were 8.66 ± 0.17, 4.21 ± 0.13, and 2.64 ± 0.66 ppm, respectively. However, formalin was not detected in the muscle of sturgeon after 8 h to 96 h formalin (25 or 50 ppm) exposure. Formalin concentrations in culture water, liver, and muscle gradually decreased with increasing time of formalin exposure. Formalin concentrations were under the limit of detection in culture water and liver tissues of sturgeon exposed to formalin for 48 h and 96 h (**Table 5**). Rainbow trout exposed to 50 ppm formalin, formalin concentrations in culture water and liver and muscle tissues gradually decreased with increasing time of formalin exposure. Formalin concentrations were under the limit of detection in culture water and liver, and muscle tissues of rainbow trout after 96h, 240 h, and 24 h formalin exposure (**Table 5**).

8. Discussion

During the years 1993 to 2000, fish culture has become one of the most important aquaculture sectors in Taiwan, with most of the product exported to global markets. Development of the sturgeon and rainbow trout industries are an important in the wider context of global food supplies. Thus, the use of drugs in the sturgeon and rainbow trout aquaculture has become very important. Formalin is widely used for disinfection and to prevent pathogen infection in veterinary medicine [12]. Since its pathological and physiological effects are well known, its use in animal husbandry has the potential to result in residues in animal tissues that could pose potential health risks to humans. Therefore, the use of formalin must be carefully considered.

Whether formalin is retained in fish tissues via gills and skin following exposure to formalin is an important question. Previously, formalin residues of 5.75 g/g in sliver barb (*Puntius gonionotus* Bleeker) muscle and 14.03 g/g in walking catfish (*Clarias batrachus* L.) were demonstrated in muscle tissues following a 50 ppm formalin bath for 24 h [19]. In other fish species, no significant differences in formalin residues in the muscle of rainbow trout (*Oncorhynchus mykiss*), coho salmon (*Oncorhynchus kisutch*), largemouth bass (*Micropterus salmoides*), and channel catfish (*Ictalurus punctatus*) were observed following exposure to formalin for 1 or 3 h, compared to those in the control group [18]. The results were also similar in olive flounder (*Paralichthys olivaceus*), Nile tilapia (*Tilapia niloticus*), and blue shrimp (*Penaeus stylirostris*) [20-22]. In this study, formalin residue in the sturgeon muscle was below the limit of detection within 1 h of formalin (25 and 50 ppm) exposure. In rainbow trout muscle, formalin residues were under the limit of detection within 8 h (25 ppm) or 24 h (50 ppm) formalin exposure. Additionally, formalin residues in the culture water and liver tissues of sturgeon and rainbow trout gradually decreased with increased time of formalin (25 or 50 ppm) exposure. These results suggest that fish species may affect the degree of formalin residue in fish muscle tissues.

Fish species may affect formalin tolerance. Cruz and Pitogo [23] reported that the LC_{50} values at 24, 48, 72, and 96 h were 322, 260, 241, and 232 ppm formalin, respectively, in milkfish (Chanos

chanos). Fajer-Ávila et al. [24] reported that the formalin LC_{50} for the bullseye puffer fish (Sphoeroides annulatus) ranged from 1,095 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} values for formalin in sturgeon and rainbow trout were 216 and 211 ppm, respectively. Additionally, other factors, such as gill function, acid-base balance, pituitary activation, water temperature, pH value, fish health, and culture filtration system, also affected formalin toxicity [25-28].

Sturgeon and rainbow trout culture and export in Taiwan are very important industries. Formalin is an effective chemotherapeutant, which is used to control certain ectoparasitic and bacterial infections in fish disease therapies [29]. Unfortunately, it is toxic to fish and its pathological and physiological effects have been previously reported. Because formalin is an effective sanitizer, it is widely used in veterinary medicine. The resulting drug residues in fish tissues are an important issue. Although the level of formalin residues in fish tissues is not detected based on the Standards of Veterinary Drug Residues in Aquatic Animal Drugs Use in Taiwan, understanding the pharmacokinetics and residues of formalin in fish tissues is necessary to guard against food and safety problems occurring in the future in Taiwan.

For a long time, formalin was used for disease prevention and therapeutically in ornamental fish in Taiwan and in salmon and trout in the USA. Many methods have been described for the determination of formalin worldwide. One method, 2,4-dinitrophenylhydrazine derivatization with solid/liquid phase extraction and HPLC, has been the major method for formalin detection in many countries [18]. In Taiwan, formalin detection was performed 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, a detection method for formalin residues in animal tissues has been lacking in Taiwan until now. Thus, the development of a simple, rapid, and reliable technique to determine the pharmacokinetics and quantify residues of formalin in animal tissues is needed. In this study, our data showed that the formalin retain time was 6.2 min in sturgeon and rainbow trout, the LOQ and LOD were 5 and 0.1 ppb, and the recoveries (%) of formalin in the spiked muscle and liver of sturgeon and rainbow trout were 94-95% and 96.5-97%, respectively. Thus, the use of HPLC with ultraviolet detection was able to satisfactorily determine formalin pharmacokinetics in the muscle and liver of sturgeon and rainbow trout.

Sturgeon and rainbow trout are some of the most important fish species and formalin is a widely used sanitizer in fish farms. A portion of any administered pharmaceutical 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 with their consumption, have become a public safety issue [9]. Therefore, the knowledge of pharmacokinetic studies of formalin in sturgeon and rainbow trout is crucial. The elimination rate of formalin in fish tissues and culture water was rapid; therefore, 25 and 50 ppm formalin are safe in Bester sturgeon and rainbow trout and the drug residue should not be of concern.

In conclusions, we have developed a method for the quantification of formalin in the cultured hybrid Bester sturgeon (*Huso huso* × *Acipenser ruthenus*) and rainbow trout (*Oncorhynchus mykiss*). Precipitation, centrifugation, and filtration of a mixture of animal tissue with acetonitrile in water and subsequent continuous solid-phase extraction provide a reliable protocol to remove co-extracting interferences (mainly proteins and lipids) from complex matrices and facilitate preconcentration of pharmaceuticals for detection. The method is reasonably sensitive (LOD: 1 ppb; LOQ: 5 ppb), accurate, and precise. The pharmacokinetics of formalin were studied in sturgeon and rainbow trout following exposure and indicated that the elimination rate of formalin in sturgeon and rainbow trout or their culture water was very rapid. To the best of our knowledge, this is the first report of pharmacokinetic studies in sturgeon and rainbow trout in Taiwan. The pharmacokinetic data for sturgeon and rainbow trout will be used to design rational dosing regimens and to assist the Taiwan government in formulating laws and regulations for formalin residues in sturgeon and rainbow trout tissues in the future. This study will demonstrate that the issue of formalin residues in fish is not as serious as previously suggested. Therefore, formalin is the most economical and effective drug for parasitic aquatic parasite treatment currently available. Additionally, this information will serve as a reference for drug selection and dosage reference to breeders and veterinarians and improve consumer protection for aquatic agricultural products.

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