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Determination of Niclosamide and its Metabolites in Liver and Muscles of Common Carp (*Cyprinus carpio*) Fingerlings

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ABSTRACT

Background: Niclosamide is a medication used to treat tapeworm infestation in animals and humans. It is also lampricide and molluscicide, and can be used in agriculture as a pesticide. In the treatment of parasitic diseases in fish, niclosamide can be used as bath or mixed with the feed. Its most important use in common carp (*Cyprinus carpio*) is for the treatment of *Bothriocephalus acheilognathi*, which is a very common parasite in this fish species. The aim of this study was to determine the concentrations of niclosamide (NIC) and its metabolite 2-chloro 4-nitro aniline (CNA) and 5-chloro salicylic acid (CSA) in the liver and muscles of common carp fingerlings.

Materials, Methods & Results: The fish for the experiment were obtained from Kapetanski Rit fish pond, and were acclimated to test conditions at $20.5 \pm 1^\circ\text{C}$. Common carps with an average mass of 60 ± 10 g were treated with niclosamide in concentration of 2 g/kg of feed during five consecutive days. The experiment was performed in two treatments: one control and niclosamide, in three replications. Each group contained of 30 fish, in 120 L polyethylene tanks. At the end of the treatment, the levels of niclosamide residues were determined using a high performance liquid chromatography (HPLC) analysis during over 13 days. The mean values of niclosamide and CNA concentrations in the muscles ranged from 27.7 µg/kg starting from the first day to <0.5 µg/kg on the 11th day and 14.2 µg/kg from the first day to <1 µg/kg on the 9th day. The CSA metabolite in muscles were <1 µg/kg during throughout the entire study. The niclosamide concentration in the liver were found to be 51.5 (30.2-61.8) µg/kg the first day and decreased proportionally to <0.5 µg/kg on the 13th day. CNA level in the liver of treated Common Carps amounted to 170.1 (157-181) µg/kg on the first day and continuously declined until the 13th day when recorded values were <1 µg/kg. The CSA concentrations in the liver reached a maximum level of 11.5 (10.1-12.8) µg/kg on the 7th day and fell to <1 µg/kg on the 13th day.

Discussion: Niclosamide use in fish is questionable, primarily due to the possible toxic effects on some aquatic organisms. In Serbia, niclosamide preparation for use in aquaculture, has been produced by Veterinarski zavod Subotica since 1984 when it was registered for the first time. Niclosamid degradation mechanism showed that the metabolism of niclosamide resulted in two main metabolites CNA and CSA. Withdrawal of niclosamide and its residues in the liver and muscle in the present investigation lasted from 9 to 13 days. This decrease in residues concentrations is expected and depends primarily on several factors such as the length and concentration of drug with which the fish is treated, biotransformation, excretion and decomposition of used drug. Niclosamide and CNA were proportionally decreased during the withdrawal time, while the CSA value increased to the seventh day although the fish during this period no longer consumed food with niclosamide, after which the value then decreased until the end of its elimination. This is also not unexpected because it is known that liver and gallbladder is a major organ for collection, storage and elimination of chemical residues. Although the treated fish received 2 mg of the niclosamide per g of feed for five consecutive days results obtained in this study indicate that the maximal residues concentrations were much lower than doses of niclosamide that each fish absorbed into the body. Data obtained during this study provided information about the concentration and withdrawal times of niclosamide and its residues CNA and CSA in the liver and muscles of common carp treated orally.

Keywords: niclosamide residues, distribution, common carp, liver, muscles.

INTRODUCTION

Niclosamide (2', 5-dichloro-4'-nitrosalicylanilide) is an anthelmintic drug belonging to the salicylanilide structural family [11], which is especially effective against cestodes. Niclosamide is used as lampricide and molluscicide [1], in agriculture as a pesticide, as well as in human and veterinary practices [2,5,8,17]. Niclosamide, along with oxyclozanide, was found to display "strong *in vivo* and *in vitro* activity against methicillin-resistant *Staphylococcus aureus* (MRSA)" [13], and suggested that may inhibit Zika virus replication *in vitro* [20]. A number of studies have established the anticancer activities of niclosamide and have identified it as a potential anticancer agent [9,10].

The drug affects tapeworms by uncoupling oxidative phosphorylation or stimulation of ATPase activity, killing tapeworms on contact [19]. Despite its general use, niclosamide is quickly metabolized in water and does not exhibit any long-term effects [3].

In the treatment of parasitic diseases in fish, niclosamide was used as bath in therapy of some monogeneans and was effective in a narrow concentration range of 0.075-0.1 mg/mL (90 min) [15]. It can also be used when mixed with the feed in order to control cestodes infestation. Its most important use in common carp (*Cyprinus carpio*) is for the treatment of *Bothriocephalus acheilognathi*. Treatment of common carp with niclosamide (3.4 g/kg in feed) for three consecutive days resulted in up to 100% elimination of tapeworms [4].

The aim of the present investigation is to determine the concentrations and withdrawal period of niclosamide residues in the muscles and liver of common carp using a high performance liquid chromatography (HPLC).

MATERIALS AND METHODS

Animals and Experiment

The fish for the experiment were obtained from Kapetanski Rit fish pond, and were acclimated to test conditions (pH 7.5; 8.1 mg/L as O₂; 483 µS/cm as electrical conductivity) at 20.5 ± 1°C. Common carp (*Cyprinus carpio*) with an average mass of 60 ± 10 g were used. During the study, the fish were divided into 4 groups of 30 fish, each in 120 L polyethylene tanks at the University of Belgrade, Faculty of Agriculture, Laboratory of Fish Nutrition. The experiment was per-

formed in two treatments: one control and niclosamide (2 g/kg of feed), in three replications. In the study, the doses of niclosamide were administered to the fish by being mixed with palletted feed for 5 days. The fish were fed an amount of food which corresponds to 3% of ichthyomasse each day. At the end of the treatment, concentrations of niclosamide and its metabolites in the muscle and liver were observed over the course of 13 days. At each sampling, 3 fish from each tank were taken. The physical and chemical properties of the water (temperature, oxygen concentration, saturation of water with oxygen, conductivity and pH) were measured daily in each tank by multifunctional measuring instrument Multi 340i by WTW¹ in combination with OxyGuard System². The behavior of the fish in the tanks, food consumption and the possible death of the fish were monitored every day. During the days when the fish were taken for sampling, the total amount of feed from that day on, was corrected to the amount of feed that was proportional to the mass of the sacrificed fish.

Fish tissue preparation

The control fish meat and liver were frozen and stored in plastic containers in the freezer, were partially thawed, weighed (about 1 g) and placed into Corex 25-mL centrifuge tubes. The meat and liver samples (n = 7) and one control were fortified with 1 mL of a 50 µg/L NIC, CSA and CNA standards using a volumetric pipette. The fortified tissue was allowed to come in contact with the chemical solution for a minimum of 30 min before extraction. Homogenized samples of the meat and liver were extracted with 3 mL of mobile phase in ultrasonic bath, during 30 min. After extraction 1 mL of the raw extract was filtrated through Cronus Syringe Filter Nylon. Clean extracts were injected to the HPLC system in a quantity of 10 µL. Control samples were extracted and cleaned in the same way.

HPLC analysis

The HPLC analyses were performed with a Thermo Ultimate 3000 model by Thermo Fisher Scientific³ coupled with a photodiode array detector set at 290 nm and Fluorescence Detector (FLD). The HPLC system was controlled by Chromeleon software. The mobile phase was pumped at a flow rate of 1 mL/min. The column oven was heated at 30°C. For analysis a C18, standard-size HPLC column (4.6 x 250 mm) was

used. Niclosamide (98.7%) and CNA (99.3%) were purchased from Dr. Ehrenstorfer GmbH⁴, while CSA (98%) was purchased from Sigma Aldrich⁵. HPLC grade Acetonitrile (ACN), Methanol and Phosphoric Acid (85%, Wt) were purchased from Fisher Scientific⁶. HPLC grade water was used; the water was filtered through White membrane filter 0.2 µm, was obtained from Lab Logistics Group GmbH⁷. The mobile phase used was acetonitrile–water with the addition of 0.1% H₃PO₄ (60:40) and detection wavelength at 290 nm for niclosamide determination. The mobile phase methanol - water with the addition of 0.1% H₃PO₄ (60:40) was used in determining CNA and CSA. Detection wavelength at 211 nm was used for CNA determination, CSA was detected in emission mode in range wavelength of 315-470 nm. The stock solution was prepared dissolving a 10 mg of NIC, CSA and CNA with methanol and amounted to 100 mL. Obtained solution was stable at 4°C for several months.

Method validation

The method determination of NIC, CSA and CNA was a modified method, prepared in accordance with ISO 17025. The validation plan included determination of precision, reproducibility, recovery, linearity, limit of detection (LOD) and limit of quantification (LOQ) [Table 1]. The meat and liver of the fish without treatment were prepared in accordance to validated the method. Samples were measured, extracted and cleaned up. After that sample were analyzed on the HPLC with UV and FLD detectors. Quantitative and qualitative analyses were done using Chromeleon software. Samples were analyzed in order to not have

any residues of NIC and its metabolites. Those samples were used for internal control and validation study as a blank. The precision of the method was evaluated by repeatability using the blank fortified with NIC, CSA and CNA injected in triplicate (50.0 µg/kg, n = 7). Extraction efficacy was calculated by recovery. Linearity of detector was tested in a range of 0.05 to 0.5 µg/kg, and was satisfactory in all ranges. LOD was calculated as a three standard deviation of baseline noise, while LOQ was calculated as a ten standard deviation of the baseline noise. The LOD values ranged from 0.5 to 1.1 µg/kg, and the LOQ varied from 1.5 to 3.7 µg/kg for NIC and its metabolites.

Statistical analysis

Data analysis was performed using Statistica 12⁸ software to determine the descriptive statistic parameters (mean, standard deviation, range).

RESULTS

The results of the distribution of niclosamide and its metabolites 2-chloro4-nitro aniline and 5-chloro salicylic acid in the liver and muscles of common carp after the fish were exposed to 20 mg/g of 100% niclosamide mixed in the feed during the five-day treatment are shown in Table 2 and Table 3. The results are presented as mean concentrations including minimum and maximum values. NIC and CNA concentrations in the liver of treated common carp were the highest on the first day after the treatment and continuously declined until the 13th day when the recorded values were below the LOD. The CSA values in the liver of the common carp increased to the seventh day after the

Table 1. Average values of precision, reproducibility, accuracy, linearity, LOQ and LOD for NIC, CSA and CAN in meat and liver of common carp.

Drug/Metabolite	Precision (%)	Reproducibility (%)	Recovery (%)	Linearity (r ²)*	LOQ (µg/kg)	LOD (µg/kg)
Meat						
NIC	3.4	14.6	88.1	0.9999	1.6	0.5
CSA	3.6	3.4	98.3	0.9998	3.3	1
CNA	3.5	3.4	94.4	0.9994	3.4	1
Liver						
NIC	13.7	15.3	116.3	0.9999	1.5	0.5
CSA	3.5	3.4	94.4	0.9999	3.7	1.1
CNA	7.7	1.8	106.1	0.9994	3.3	1

*r: correlation coefficient.

Table 2. Mean concentrations (µg/kg) of NIC, CNA and CSA in the liver from carp exposed to 2 g/kg of niclosamide for selected withdrawal times.

Withdrawal time	NIC	CNA	CSA
	X* ± SD** (Ranges)	X ± D (Ranges)	X ± SD (Ranges)
Control	<0.5	<1	<1
Days			
1	51.5 ± 9.5 (30.2-61.8)	170.1 ± 7.6 (157-181)	0.8 ± 0.1 (0.7-0.9)
3	41.6 ± 8.2 (29.7-56.1)	64.6 ± 20.1 (36-92)	1.3 ± 0.4 (1-2.1)
7	3.0 ± 0.6 (2.1-3.7)	28.1 ± 5.9 (18-34)	11.5 ± 1.0 (10.1-12.8)
9	1.6 ± 0.6 (0.9-2.8)	15.6 ± 3.3 (11-20)	10.2 ± 0.3 (10-10.9)
11	0.7 ± 0.9 (0.5-1)	8.2 ± 0.7 (6.8-9.2)	2.6 ± 1.4 (1-4.9)
13	<0.5	<1	<1

*X: mean; **SD: standard deviation.

end of treatment, when they reached a maximum value of 12.8 µg/kg, after which it started to decrease by the end reaching below 1 µg/kg on the 13th day.

The concentration of niclosamide and CNA in the muscles were lower than in the liver and were maintained up to 11 days when the values were below 0.5 µg/kg for niclosamide and on the 9th day for CNA, CSA metabolite in muscles were under the LOD values.

DISCUSSION

Although niclosamide is used for the treatment of most tapeworm infestations in animals and humans, its use in fish is questionable, primarily due to the possible toxic effects on some aquatic organisms [3,12]. In Serbia, niclosamide preparation for use in aquaculture, has been produced by Veterinarski zavod Subotica since 1984 when it was registered for the first time. Its main indication was to treat *Bothriocephalus acheliognati* presents in young cyprinid fishes, primarily common carp. In recent years it has been withdrawn from the market and since there is no effective alternative, it has resulted in it being used as an unregistered drug.

Niclosamid degradation mechanism has been reported by Rotzinger *et al.* [14] and Zaazaa *et al.* [21]. They showed that the metabolism of niclosamide resulted in two main metabolites CNA and CSA. Me-

tabolism of niclosamide by hydrolysis, reduction, or conjugation with glucuronic acid has been reported in fish, rats, cestodes, nematodes, mouse and sheep [7].

Withdrawal of niclosamide and its residues in the liver and muscle in the present investigation lasted from 9 to 13 days. This decrease in residues concentrations is expected and depends primarily on several factors such as the length and concentration of drug with which the fish is treated, biotransformation, excretion and decomposition of used drug [6]. Niclosamide and CNA were proportionally decreased during the withdrawal time, while the CSA value increased to the seventh day although the fish during this period no longer consumed food with niclosamide, after which the value then decreased until the end of its elimination. This is also not unexpected because it is known that liver and gallbladder is a major organ for collection, storage and elimination of chemical residues [18]. Similar results were reported by Dawson [6] who investigated concentration of niclosamide residues in bile of rainbow trout, coho salmon, channel catfish and largemouth bass after bath exposure. He also recorded an increase in concentration of residues in the bile after the fish were transferred to clean water and then a slow decrease as a result of partly reabsorption and recycling from bile secreted into the digestive tract.

Table 3. Mean concentrations (µg/kg) of NIC, CNA and CSA in the muscles from carp exposed to 2 g/kg of niclosamide for selected withdrawal times.

Withdrawal time	NIC	CNA	CSA
	X* ± SD** (Ranges)	X ± SD (Ranges)	X ± SD (Ranges)
Control	<0.5	<1	<1
Days			
1	27.7 ± 4.6 (20.9-37.2)	14.2 ± 1.9 (12.2-17.2)	<1
3	15.7 ± 2.5 (12.1-20.1)	6.7 ± 1.1 (5.6-8.7)	<1
7	2.8 ± 1.2 (1.1-5.1)	1.4 ± 0.5 (1-2.1)	<1
9	1.2 ± 0.6 (0.5-2.2)	<1	<1
11	<0.5	<1	<1
13	<0.5	<1	<1

*X: mean; **SD: standard deviation.

Although the treated fish received 2 mg of the niclosamide per g of feed for five consecutive days (fish weight 60 g consume 3% of ichthyomass a day which result in 1.8 g of daily consumed feed or 3.6 mg of niclosamide per day i.e 18 mg for five days) results obtained in this study indicate that the maximal residues concentrations were much lower than doses of niclosamide that each fish absorbed into the body. It should be noted that the use of niclosamide in carp fingerlings cannot have harmful effects on people as potential consumers, because this is not older fish aimed for consumption.

There is very little research relating to the distribution of niclosamide in fish, especially after oral administration of the drug. Schreier *et al.* [16] developed a method that may be used for monitoring niclosamide residues in rainbow trout and channel catfish tissues that have been exposed to niclosamide lampricide treatments or to obtain additional residue chemistry data required for reregistration of niclosamide by the EPA. Data obtained during this study provided information about the concentration and withdrawal times of niclosamide and its residues CNA and CSA in the liver and muscles of common carp treated orally.

CONCLUSIONS

Niclosamide residues in common carp liver and muscle were successfully determined using the HPLC instrument equipped with a UV and FLD detector. According to the presented investigation of concentration

of niclosamide and its residues in the liver and muscles of common carp fingerlings it can be concluded that its elimination ends from for 9 to 13 days. However, it is necessary to continue to work on studies dealing with distribution, elimination and toxicity of niclosamide and its metabolites in common carp to justify its use in therapy of *Bothriocephalus acheliognati* in order to renew its registration.

MANUFACTURERS

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Ethical approval. All procedures, treatments and animal care were in compliance with the Animal welfare law (Official Gazette of Republic Serbia, Number 41/2009). Ministry of Agriculture and Environmental Protection of Serbia, Veterinary Office, approved this experiment (Rescript Number: 323-07-04167/2017-05).

Declaration of interest. The authors report no conflicts of interest. The authors alone are responsible for the content and writing of paper.

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