EVIDENCE OF ^{14}C-FURAZOLIDONE METABOLITE BINDING TO THE HEPATIC DNA OF TROUT

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SUMMARY

Furazolidone (FZ) is a nitrofuran drug commonly used in aquaculture. In the present study, [methylidene-^{14}C]-FZ or [oxazolone-4,5-^{14}C]-FZ was offered to rainbow trout *(Oncorhyncus mykiss)* in medicated feed at a daily dose of 135 mg/kg b. wt. for 10 days. The trout were sacrificed at specific time points post-dosing and the liver removed for DNA-bound ^{14}C characterization. Both forms of the ^{14}C-labelled FZ were converted by trout to reactive metabolite(s) which bound irreversibly to the hepatic DNA. The amount of ^{14}C bound to the hepatic DNA increased with post-dosing time and was higher in trout pretreated with [methylidene-^{14}C]-FZ than in trout pretreated with [oxazolone-4,5-^{14}C]-FZ. The identity of the FZ reactive metabolite(s) remained to be elucidated. However, a part of the FZ reactive metabolite(s) could be released as 3-amino-2-oxazolidone by acid hydrolysis. An appreciable amount of ^{14}C was also found to bind irreversibly with the hepatic DNA of trout following an i.v. injection of [oxazolone-4,5-^{14}C]-FZ. Results of these studies indicate that FZ is metabolized by trout to a reactive metabolite(s) which binds irreversibly to the DNA of trout liver.

KEY WORDS

furazolidone, DNA, rainbow trout

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INTRODUCTION

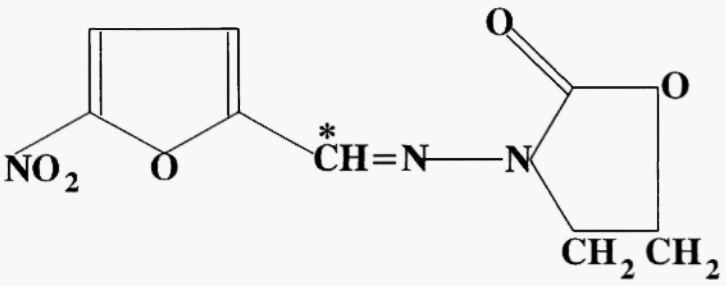
Furazolidone (FZ), N-(5-nitro-2-furfurylidene)-3-amino-2-oxazolidone, is a nitrofuran drug used frequently as a growth promoter and antimicrobial feed additive for disease control in aquaculture /1/. The use of FZ to treat bacterial diseases in farmed fish has received considerable attention in recent years since nitrofuran drugs have been shown to be mutagenic and carcinogenic in biological test systems /2-4/. Nitrofuran drugs must be metabolized by animals to reactive metabolites which bind irreversibly with RNA and DNA /5/ before inducing the genotoxic effects /6-8/.

Despite a renewed interest in the genotoxicity of FZ, very little is known of the interactions between FZ and the cellular macromolecules such as proteins, DNA and RNA of fish. This is probably due to the fact that an accurate, sensitive and specific method for FZ reactive metabolite isolation is presently unavailable /9/. A recent study from our laboratory has shown that a high level of ^{14}C is bound to the hepatic proteins of trout following ^{14}C-FZ medicated feed treatments /10/. This finding has prompted us to investigate whether ^{14}C binding could also be demonstrated in the hepatic DNA of ^{14}C-FZ treated trout. Since the use of FZ in food-producing animals has been banned by the US Food and Drug Administration /11/, additional information on the binding of FZ reactive metabolites to the hepatic DNA of fish would be very useful for a safety reevaluation of FZ as an aquaculture drug. The present paper describes the *in vivo* binding of FZ reactive metabolite(s) to the hepatic DNA of rainbow trout *(Oncorhyncus mykiss)* and the chemical characteristics of a component of the reactive metabolite structure.

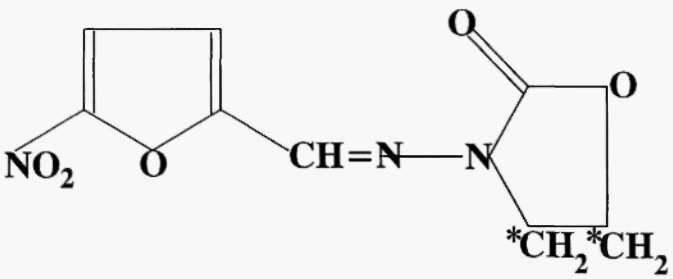
MATERIALS AND METHODS

Chemicals

Unlabelled FZ was obtained from Sigma Co. (St. Louis, MO). The chemical purity of unlabelled FZ, as shown by HPLC, was more than 98%. Two differently labelled ^{14}C-FZ compounds were custom-synthesized for the study by Amersham Canada Ltd. (Oakville, ON); ^{14}C-FZ was labelled either at the methylidene moiety (238 μCi/mg) or at the 4,5-oxazolidinone moiety (34 μCi/mg) of the FZ molecule (Fig. 1). The radiochemical purities of the [methylidene-^{14}C]-FZ and



[Methylidene-^{14}C]-FZ



[Oxazolone-4,5-^{14}C]-FZ

Fig. 1: Structure of furazolidone. Asterisks indicate positions of the ^{14}C label.

[oxazolone-4,5-^{14}C]-FZ were shown by HPLC and liquid scintillation counting to be more than 98%. 3-Amino-2-oxazolidinone (AOZ) and 2-nitro-benzaldehyde (NBA) were obtained from Aldrich Chemical Co. (Milwaukee, WI). Ribonuclease A (RNase A), ribonuclease T1 (RNase T1), and proteinase K were purchased from Sigma Co. (St. Louis, MO). All other chemicals were reagent grade and of the highest purity available.

3{[(2-Nitrophenyl)-methylene]amino}-2-oxazolidone (NPAOZ) was synthesized chemically at our laboratory according to the procedure reported by Hoogenboom *et al.* /12/. Briefly, NBA and

AOZ were dissolved in an acetonitrile/DMSO solution and allowed to react at 50°C for 24 h. The reaction product NPAOZ was purified by preparative HPLC.

Fish

Rainbow trout (*Oncorhyncus mykiss*) weighing about 300 g were purchased from the Spring Valley Trout Farm (Langley, BC). The fish were transported to the Aquatic Facility of Simon Fraser University and quarantined in a holding tank for at least 1 week before use. The trout were kept in flowing, dechlorinated freshwater under the following conditions: temperature, 10 ± 2°C; pH, 6.0-7.0; hardness, 5-10 mg/l; and dissolved oxygen, 9 ± 2 mg/l. The fish were fed twice daily at 9.0 a.m. and 9.0 p.m. with 3.5 mm extruded pellets (Moore-Clarke Co., Vancouver, BC).

Preparation of ^{14}C-FZ medicated feed

^{14}C-FZ medicated feed was prepared by mixing 25 g of commercial fish feed (Moore-Clark Co., Vancouver, BC) with 35 ml of acetone containing 500 mg of ^{14}C-labeled FZ (specific activity 0.57 μCi/mg). The acetone was removed using a rotary evaporator. ^{14}C-FZ medicated feed was prepared in batches. Different batches of the medicated feed were combined, mixed thoroughly and stored at 2°C before use. HPLC analysis showed that ^{14}C-FZ in the medicated feed was stable chemically for at least 30 days under the storage conditions.

Treatment of trout with ^{14}C-FZ

Trout were removed randomly from the holding tank and put into two separate 200-l experimental tanks. One group of trout was fed [methylidene-^{14}C]-FZ medicated feed at a daily dose of 135 mg/kg body weight for 10 days. The other group was fed similarly with [oxazolone-4,5-^{14}C]-FZ medicated feed. At the conclusion of a 10-day dosing period, both groups of fish were given non-medicated feed for an additional 5 days. At specific post-dosing time points (0, 48 and 120 h), three trout were removed randomly from each of the experimental tanks and frozen immediately in liquid nitrogen. In a second experiment, the effects of a single i.v. dose of ^{14}C-FZ on the formation of DNA-bound ^{14}C in the liver were studied. Each trout was given a bolus injection of [oxazolone-4,5-^{14}C]-FZ (1 μCi/g) *via* the caudal

vein. Three trout were sacrificed at 24 h and 48 h post-dosing and frozen immediately in liquid nitrogen. The fish were kept frozen until analysis.

Determination of total radioactivity in liver

Trout were thawed at room temperature. The abdominal wall of the trout was opened with a surgical knife and the liver removed. A 50% w/v liver homogenate was prepared in distilled water using a Polytron homogenizer (Brinkman Instrument, NY). An aliquot (0.5 ml) of the liver homogenate was combusted in a Model OX 300 Biological Material Oxidizer (R.J. Harvey Instrument Corp., Hillsdale, NJ). The $^{14}CO_2$ evolved was trapped in Carbon ^{14}C Cocktail (R.J. Harvey Instrument Corp., Hillsdale, NJ) and the radioactivity trapped was measured by a Beckman LC 8000 Liquid Scintillation Counter. Recovery of radioactivity from the Biological Material Oxidizer was estimated with a ^{14}C standard (Du Pont Co., Wilmington, DE) and found to be more than 97.5%.

Isolation of DNA from the liver

About 1 g of the liver was homogenized in 10 ml Tris buffer (pH 8.2; Tris-HCl 10 mM, NaCl 400 mM and EDTA 2 mM) containing 1% (w/v) sodium dodecyl sulfate (SDS) buffer. The liver homogenate was incubated with proteinase K (0.2 mg/ml) at 37°C for 4 h. The mixture was transferred to a test tube containing an equal volume of Tris-EDTA buffer (pH 7.5; 10 mM Tris-HCl and 0.2 mM EDTA) previously saturated with phenol. The test tube was centrifuged at 10,000 *g* for 10 min. The phenolic phase was discarded. The remaining aqueous phase was re-extracted with chloroform-isoamyl alcohol (24:1 v/v) and centrifuged at 10000 *g* for 10 min. DNA was precipitated from the aqueous phase by two volumes of absolute ethanol at room temperature. The DNA precipitates were collected using a pipette tip and redissolved in 2 ml Tris-EDTA buffer. The DNA solution was mixed with RNase A (10 mg/ml) and RNase T1 (250 U/ml) and incubated at 37°C for 30 min. After incubation, proteinase K (50 mg/ml) was added to the mixture and incubated at 37°C for an additional 1 h. The DNA was precipitated and purified by the spermine method /13/. Further DNA purification was carried out by equilibrium centrifugation in a cesium chloride-ethidium bromide gradient. The

DNA fractions were collected from the gradient and dialyzed against Tris-EDTA buffer at 4°C. DNA concentration was estimated spectrophotometrically in a 1 cm optical-path cuvette at 260 nm with the assumption that one absorption unit was equivalent to 50 mg/ml DNA /14,15/.

Determination of DNA-bound ^{14}C

The DNA solutions were adjusted to 1 mg/ml concentration with Tris-EDTA buffer before being combusted in a Biological Material Oxidizer. The $^{14}CO_2$ evolved was trapped in 20 ml Carbon ^{14}C Cocktail and the radioactivity trapped was measured by a Beckman Liquid Scintillation Counter, as described above.

Preliminary characterization of DNA-bound ^{14}C

Purified DNA was dissolved in deionized water (5 mg/ml) and incubated with 0.1 M HCl and 5 mM NBA at 37°C. At the conclusion of an 18-h incubation, the incubation mixture was extracted with 2 ml of ethyl acetate. The extraction was repeated once. The ethyl acetate extracts were combined and evaporated down to dryness under a gentle stream of nitrogen. The residues were redissolved in 0.5 ml acetonitrile/water (1:1, v/v). An aliquot (50 ml) of the acetonitrile/water solution was injected into a HPLC system consisting of two Model 510 Waters Liquid Chromatograph pumps (Waters Chromatography Division, Millipore Corp., Milford, MA) and a Zorbax C_8 column (7.5 μm, 2.7 x 30 cm; Chromatographic Specialties Inc., Brockville, ON) preceded by a guard column with the same packing. The acetonitrile/water (1:1, v/v) mobile phase was delivered isocratically at a flow rate of 1.0 ml/min. The eluant was monitored with a Waters Associates Lambda-Max Model 481 LC Spectrophotometer set at 254 nm wavelength. The detection limit of the HPLC analysis was set at 5 times the mean background of the liver extract from untreated trout. The HPLC eluant fractions corresponding to the retention time of the standard NPAOZ peak were collected, combined and scanned by a Spectronic 3000 Array Spectrophotometer (Milton Roy; Rochester, NY) from 230 nm to 395 nm. The ultraviolet spectrum obtained was compared with that of the standard NPAOZ solution recorded under identical conditions.

RESULTS

Figure 2A shows the time course of ^{14}C binding to hepatic DNA of trout following [methylidene-^{14}C]-FZ or [oxazolone-4,5-^{14}C]-FZ medicated feed treatments. ^{14}C binding to DNA was found to increase with post-dosing time, indicating the involvement of reactive metabolite(s) of FZ in the formation of DNA-bound ^{14}C. The amount of ^{14}C bound DNA was higher in trout pretreated with [methylidene-^{14}C]-FZ than in trout pretreated by [oxazolone-4,5-^{14}C]-FZ. The time course profiles of hepatic DNA-bound ^{14}C in trout pretreated by either of these ^{14}C-FZ compounds are very similar (Fig. 2A); they increased with the post-dosing time. In contrast, the time course profiles of the protein-bound ^{14}C were different; they decreased with the post-dosing time (Fig. 2B).

The formation of DNA-bound ^{14}C was also examined in the liver of trout after injecting [oxazolone-4,5-^{14}C]-FZ i.v. Total ^{14}C, DNA-bound ^{14}C, and protein-bound ^{14}C were all found to increase with the post-dosing time (Fig. 3A,B).

A part of the DNA-bound ^{14}C in the liver of trout could be released by acid hydrolysis. This was demonstrated by hydrolyzing the hepatic DNA with acid and extracting the hydrolysate with ethyl acetate. HPLC analysis of the ethyl acetate extract showed two chromatographic peaks with retention times identical to those of NPAOZ (5.89 min) and NBA (8.83 min) standards (data not shown). The NPAOZ peak represented the NBA derivative of AOZ which was released from the FZ reactive metabolite(s) bound to the DNA. The NBA peak represented the unreacted chemical added initially to the incubation mixture. The UV spectrum of the NPAOZ peak was also found to be identical to that of the authentic NPAOZ reported previously /10/. However, the amount of AOZ released represented only 30-40% of the total ^{14}C bound to the DNA of the liver.

DISCUSSION

Results of the present study indicate that a significant amount of ^{14}C is bound irreversibly to the hepatic DNA of rainbow trout following treatments with ^{14}C-FZ *via* the oral or the i.v. route. Two differently labelled ^{14}C-FZ compounds were used in the study to help to elucidate the mechanism of DNA binding. Our results show that ^{14}C binding to

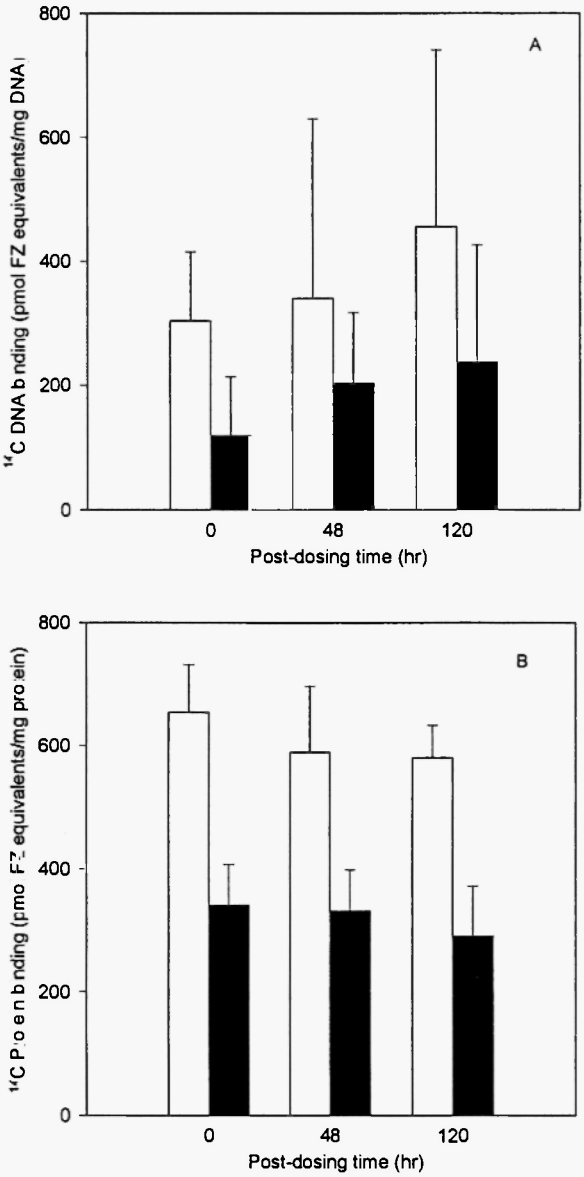


Fig. 2: Time course of DNA-bound ^{14}C in the liver of trout following ^{14}C-FZ medicated feed treatments. Results are mean ± SD of three trout. The open and filled bars are [methylidene-^{14}C]-FZ and [oxazolone-4,5-^{14}C]-FZ, respectively. **A**: DNA-bound ^{14}C. **B**: Protein-bound ^{14}C. **B** was adapted from our previous experiments /10/.

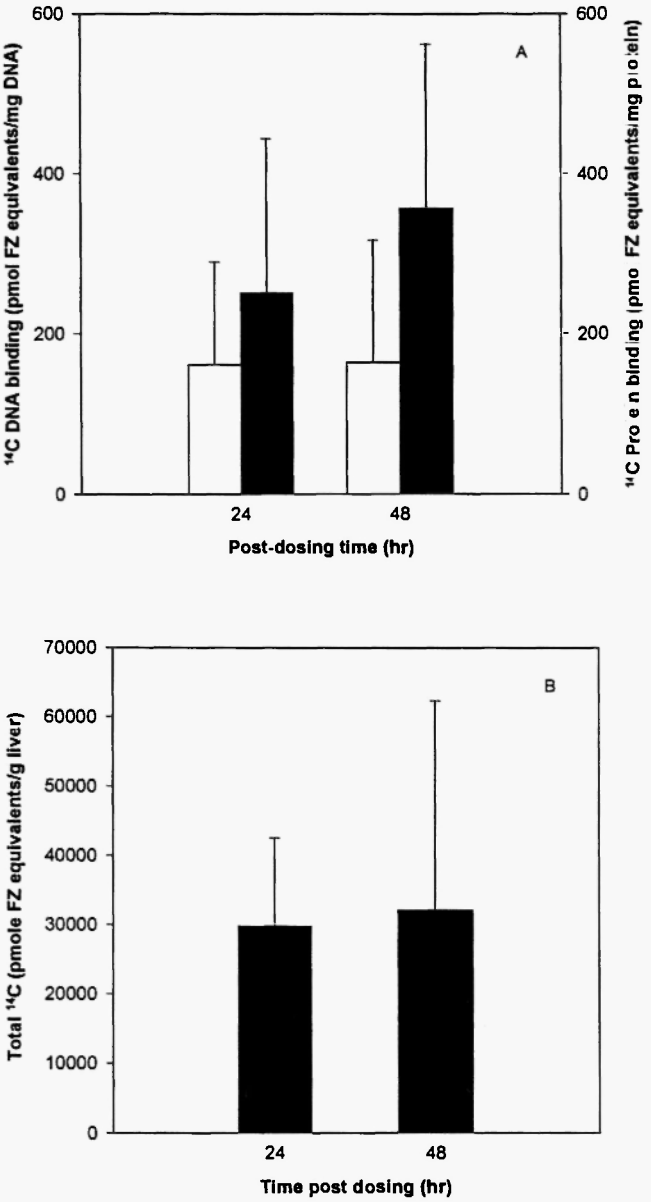


Fig. 3: Formation of hepatic DNA-bound ^{14}C in the liver of trout after intravenous administration of [oxazolone-4,5-^{14}C]-FZ. Results are mean ± SD of three trout. **A**: Open and filled bars represent DNA- and protein-bound ^{14}C-FZ, respectively. **B**: Filled bars represent total ^{14}C in the liver.

the DNA is determined by the administration route as well as the form of ^{14}C-FZ used in the study. About 160 pmol equivalents of FZ were bound to each mg of DNA at 2 days post-dosing after injecting trout with [oxazolone-4,5-^{14}C]-FZ. In contrast, about 300-450 pmol equivalents of FZ were found to bind with each mg of DNA after feeding trout with [oxazolone-4,5-^{14}C]-FZ medicated feed for 10 days. Moreover, a lower level of DNA-bound ^{14}C was found in the liver of the [oxazolone-4,5-^{14}C]-FZ treated trout as compared to that of the [methylidene-^{14}C]-FZ treated trout. These findings are consistent with the results of the protein-bound ^{14}C study reported previously /10/. A lower level of hepatic DNA-bound ^{14}C in the [oxazolone-4,5-^{14}C]-FZ treated trout as compared to the [methylidene-^{14}C]-FZ treated trout is probably related to the hydrolysis of the azomethine bond, since this results in a loss of the ^{14}C label from the ^{14}C-FZ molecule /6,16/.

The present study provides additional information on the *in vivo* binding of FZ reactive metabolite(s) to the hepatic macromolecules of trout. Although the chemical identity of the DNA-bound ^{14}C still remains to be determined, the reactive metabolite(s) most likely possesses an AOZ moiety which can be released by acid hydrolysis. Results of the present study also render further support to the findings that nucleic acids are the target sites of nitrofuran drug toxicity /8,17/.

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REFERENCES

1. Herman RL, Bullock GL. Antimicrobials and fish: A review of drugs used to treat bacterial diseases of channel catfish and rainbow trout. Vet Hum Toxicol 1986; 28: 11-17.
2. McCalla DR. Mutagenicity of nitrofuran derivatives: review. Environ Mut 1983; 5: 745-765.
3. Ali BH. Pharmacology and toxicology of furazolidone in animals: some recent research. Gen Pharmac 1989; 20: 557-563.
4. Vroomen LHM, Berghmans MCJ, Bladeren PJ, Groten JP, Vissink CJ, Kuiper HA. In vivo and in vitro metabolic studies of furazolidone: A risk evaluation. Drug Metab Rev 1990; 22: 663-676.

5. Cohen MN. Toxicity and carcinogenicity of nitrofurans. In: Bryan GB, ed. Carcinogens - A Comprehensive Survey, Vol. 4, Nitrofurans: Chemistry, Metabolism, and Carcinogenesis. New York: Raven Press, 1983; 171-231.
6. Basa J. Inter-strand cross-linking of *Vibrio cholerae* DNA induced by furazolidone: a quantitative assay by four simple methods. Mut Res 1995; 327: 5-15.
7. McCalla DR, Voutsinos D. On the mutagenesis of nitrofurans. Mut Res 1974; 26: 3-16.
8. Chatterjee SN, Benerjee SK, Pal AK, Basak J. DNA damage, prophage induction and mutation by furazolidone. Chem Biol Interact 1983; 45: 315-326.
9. Swaminathan S, Lower GM. Biotransformation and extraction of nitrofurans. In: Bryan GB, ed. Carcinogens - A Comprehensive Survey, Vol. 4, Nitrofurans: Chemistry, Metabolism, and Carcinogenesis. New York: Raven Press, 1978; 59-97.
10. Law FCP, Meng J. Binding of ^{14}C-furazolidone metabolites to the muscular and hepatic proteins of trout. Food Additiv Contam 1996; 13: 199-209.
11. FDA (Food and Drug Administration). Nitrofurans; Withdrawal of approval of new animal drug applications; final rule; final decision following a formal evidentiary public hearing. Federal Register 56, No. 164, 41902-42912, 1991.
12. Hoogenboom LAP, Kammen M, Berghmans MCJ, Koeman JH, Kuiper HA. The use of pig hepatocytes to study the nature of protein-bound metabolites of furazolidone: a new analytical method for their detection. Fd Chem Toxicol 1991; 29: 321-328.
13. Ross JA, Nelson GB, Holden KL. DNA isolation from small tissue samples using salt and spermine. Nucleic Acids Res 1992; 19: 6053-6059.
14. Varanesi U, Stein JE, Hom T. Covalent binding of benzo[*a*]pyrene to DNA in fish liver. Biochem Biophys Res Comm 1981; 103: 780-787.
15. Zhang Q, Suorsa-Super K, Curtis LL. Temperature modulated Aflatoxin B1 hepatic disposition and formation and persistence of DNA adducts in rainbow trout. Toxicol Appl Pharmacol 1992; 113: 253-259.
16. Tatsumi K, Nakabeppu H, Takahashi Y, Kitamura S. Metabolism in vivo of furazolidone: evidence for formation of an open-chain carboxylic acid and α-ketoglutaric acid from the nitrofuran in rats. Arch Biochem Biophys 1984; 234: 112-116.
17. Klemencic J, Wong CY. Mutagenicity of nitrofurans. In: Bryan GB, ed. Carcinogens - A Comprehensive Survey, Vol. 4, Nitrofurans: Chemistry, Metabolism, and Carcinogenesis. New York: Raven Press, 1978; 99-131.