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Disposition of 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone, a cyano-metabolite of furazolidone, in furazolidone-treated grouper

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*The cyano-metabolite of furazolidone (FZ), 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone, was isolated from the mixture of FZ incubated with the post-9000*g *hepatic supernatant of grouper. Its structure was confirmed by mass spectrometric and nuclear magnetic resonance spectroscopic studies. Thereafter, the disposition of the cyano-metabolite in the orange-spotted grouper (*Epinephelus coioides*) after oral and bath treatment with FZ was investigated. Qualitative and quantitative analyses of cyano-metabolite in the fish were performed by high-performance liquid chromatography. Mean recoveries of the metabolite in serum, muscle, liver and kidney were 99.8 ± 4.1, 98.6 ± 3.5, 53.1 ± 7.4 and 64.0 11.4%, respectively. Cyano-metabolite was mainly distributed in the serum and muscle rather than in the liver and kidney. After oral treatment of FZ, the peak cyano-metabolite concentrations, 167.2 ng ml^{-1} in serum and 283.2 ng g^{-1} in muscle, were reached at 5.1 and 6.7 h, respectively. The elimination half-life of cyano-metabolite was 4 h. During 24-h bath treatment of FZ, the maximum concentrations of cyano-metabolite, 258 ng ml^{-1} in serum and 204 ng g^{-1} in muscle, were found at 0.25 and 6 h, respectively. The half-life of cyano-metabolite was 0.5 h after transferring the fish to fresh seawater.*

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Keywords: distribution, elimination, cyano-metabolite, furazolidone, grouper, marine fish

Introduction

Furazolidone (FZ, figure 1) is the major nitrofuran drug widely used as an antibacterial agent in livestock and aquaculture industries. Because of its mutagenic and carcinogenic properties, drug residues should be avoided in edible tissues of animals treated with the drug. Earlier studies showed that no residues of FZ could be detected in edible tissues of fish 1 day after dosing (Sugimoto *et al.* 1979, Plakas *et al.* 1994, Guo *et al.* 2002). However, it was reported that radioactivity could still be detected in tissues of catfish 7 days after oral administration of ^{14}C-FZ (Plakas *et al.* 1994). FZ is degraded rapidly and extensively *in vivo*. Reduction of the nitro group, resulting in the formation of an open-chain cyano-metabolite, is an important metabolic pathway of nitrofurans (Swaminathan and Lower 1978). Some metabolic fates of FZ were examined in rats, swine, eel, trout and *Escherichia coli* (Tatsumi *et al.* 1981, Abraham *et al.* 1984, Nakabeppu and Tatsumi 1984, Vroomen *et al.* 1987a–c, Hoogenboom *et al.* 1989, Law *et al.* 1992, Law and Meng 1996). The cyano-metabolite 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone (figure 1), plays an important role among the major metabolites of FZ for its covalent binding with protein and DNA *in vivo* (Vroomen *et al.* 1986).

Marine fish aquaculture is an important industry in Asia, and grouper is one of the high-value species being cultured. Bacterial diseases are one of the problems encountered in grouper culture, resulting in the use of antibiotics for prophylactic and therapeutic treatments. However, information about the retention of metabolites of antibacterial drugs (e.g. FZ) in marine fishes is not available. In this study, the cyano-metabolite was biotransformed in the grouper

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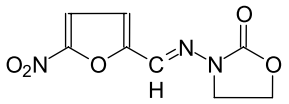
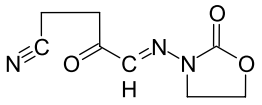


furazolidone



3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone

Figure 1.

hepatic supernatant incubated with FZ and its structure confirmed by nuclear magnetic spectroscopy and mass spectrometry. Thereafter, disposition of the cyano-metabolite in the orange-spotted grouper (*Epinephelus coioides*) treated with FZ by oral and bath administration was studied. Antibacterial activities of cyano-metabolite and its parent FZ were also compared using a susceptibility test.

Materials and methods

Chemicals

FZ, nicotinamide adenine dinucleotide phosphate (NADP), the reduced form of NADP (NADPH), glucose 6-phosphate (monopotassium salt) and tricaine methanesulphonate (MS-222) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Mueller-Hinton broth was purchased from Difco Laboratories (Sparks, MD, USA). All chemicals used were analytical or HPLC grade.

Biosynthesis of cyano-metabolite using grouper liver

Grouper livers were rapidly excised, rinsed and homogenized in 2 vols (v/w) of phosphate-buffered saline, pH 7.4, containing 1.15% KCl (PBS-KCl) with a Biomixer (Nihonseiki Kaisha, Tokyo, Japan) in an ice-cold water bath. The homogenate was centrifuged at 9000*g* for 20 min and the hepatic supernatant was inoculated in a reaction medium containing 10 mM glucose 6-phosphate, 5 mM $MgCl_2$, and 0.4 mM cofactor (NADP or NADPH) in PBS-KCl. The ratios of hepatic supernatant to the incubation medium were different depending on the final liver concentrations listed in table 1. Metabolic incubations were initiated by the addition of various amount of FZ in dimethyl formamide. Incubations were performed at 37°C under anaerobic (N_2) or aerobic (air) conditions. After different incubation times, aliquots were taken for analysis and purification of the metabolite.

Table 1. Formation of 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone (cyano-metabolite) from furazolidone (FZ) using grouper hepatic supernatant under various independent factors at 37°C after a 1 h incubation.

Independent Factors				Formation of cyano-metabolite (nmol g^{-1} liver)
FZ ($\mu g\,ml^{-1}$)	Incubation condition	Liver ($g\,ml^{-1}$)	Cofactor	
25	aerobic	0.8	NADP	30.3
50	aerobic	0.8	NADP	59.2
100	aerobic	0.4	NADP	85.6
100	anaerobic	0.4	NADP	326.7
100	anaerobic	0.4	NADPH	404.5
100	anaerobic	0.5	NADPH	278.9
75	anaerobic	0.1	NADPH	415.7
75	anaerobic	0.2	NADPH	574.3
75	anaerobic	0.3	NADPH	632.0
75	anaerobic	0.4	NADPH	150.8
75	anaerobic	0.5	NADPH	51.5

Isolation and identification of the cyano-metabolite

Aliquots of the incubated mixture were extracted three times with 2 vols (v/v) of ice-cold ethyl acetate. The combined ethyl acetate extract was filtered through filter paper topped with an anhydrous sodium sulfate layer. The filtrate was then evaporated to dryness at 37°C under vacuum. The residue was then redissolved in 30 ml acetonitrile and extracted with 40 ml *n*-hexane to remove fat. The acetonitrile layer was separated from *n*-hexane and then evaporated to dryness at 37°C under vacuum. The defatted residues were again redissolved in 0.5–1 ml acetonitrile–water mixture (20:80) and then filtered through a 0.2-μm membrane filter before HPLC separation.

The HPLC system consists of a Waters 600 controller, a Waters 717 Plus autosampler and a Waters 996

photodiode array (PDA) detector with the Millennium software (Waters Corp., Milford, USA) for data processing and output. A semipreparative reversed-phase, 6-μm Prep. Nova-Pak HR C_{18} column, 7.8 mm × 30 cm, was used for sample purification with a Nova-Pak C_{18} guard column (Waters Corp.) attached ahead. The mobile phase was an acetonitrile–water solution (20:80) at flow rate of 2 ml min^{-1}. The wavelength of PDA detector was set in the range 200–500 nm. The injection volume for preparative separation was 200 μl.

The fractions with cyano-metabolite were monitored and collected by their chromatographic behaviour and ultraviolet spectra and were freeze-dried under vacuum. The residue of the cyano-metabolite was redissolved in mobile phase solution and chromatographed again isocratically to determine its purity and then subjected for further spectroscopic and mass spectrometric analyses.

Infrared (IR) spectra of the cyano-metabolite in KBr were recorded with a Nicolet (Madison, WI, USA) 510P FT-IR. Proton nuclear magnetic resonance (^{1}H-NMR) spectra of cyano-metabolite were obtained with a Bruker (Karlsruhe, Germany) DMX-500 MHz FT-NMR in $CDCl_3$ using $Si(CH_3)_4$ as internal standard. Electron-impact mass (MS) spectra were obtained with a Finnigan (San Jose, CA, USA) MAT 95s mass spectrometer.

Oral and bath administration of FZ

The studies were conducted at the Tungkang Marine Laboratory of the Taiwan Fisheries Research Institute. Orange-spotted grouper, mean body weight 678 ± 76 g, were acclimatized for 2 weeks in cages in a 40 × 15 × 1.5 m pond with flow-through seawater of 30 ppt salinity, pH 7.9. The experiments were performed at a water temperature of 30 ± 2°C. The fish were fed with sand borer (*Sillago sihama*) twice a day. They were starved 1 day before FZ treatments.

The oral dose of FZ was administered in feed mixture at 50 mg kg^{-1} body weight. Before drug administration the fish were anaesthetized in well-aerated water containing 200 mg l^{-1} MS-222 for 2–3 min and then weighed. FZ was mixed with the eel formulate feed, which was force-fed orally to the fish. Four fish were sampled for analysis before drug administration and at different intervals (1, 3, 6, 24, 48 and 72 h) after administration.

For bath treatment, fish were exposed in FZ solution at a dose of 10 ppm in a 500-litre FRP tank. After 24 h of bath treatment, the fish were transferred to fresh seawater. Four fish were sampled at different intervals during the bath (0, 0.25, 1, 3, 6 and 24 h) and post-bath periods (0.25, 0.5, 1, 24, 48 and 72 h). Both bathing and post-bathing treatments were performed at the same room temperature.

The blood of each fish sample was drawn from the caudal vein. The liver, kidney and muscle tissues were also collected separately. The blood was allowed to clot, and was centrifuged at 3000 rpm under 4°C for 20 min. The serum, liver, kidney and muscle were stored at −80°C until analysed.

Analysis of cyano-metabolite in fish

Cyano-metabolite concentrations in fish tissues were determined by a reversed-phase HPLC with PDA detector. Serum samples, 2 ml each, were agitated three times with 30 ml ice-cold ethyl acetate and extracts were collected. Muscle (5 g), liver (5 g) and kidney (2 g) samples were homogenized for 2 min in 30 ml ice-cold ethyl acetate using Bio-mixer, and after centrifugation at 3000 rpm for 5 min the supernatant was collected. This procedure was repeated three times and the collected supernatants from each tissue samples were combined into one sample. The ethyl acetate extracts were then filtered, defatted and analysed according to the method for cyano-metabolite isolation described above, except for tissue samples wherein residues were redissolved in 0.5 ml mobile phase solution after drying and 100 μl sample was injected to HPLC for analysis.

Antibacterial activity

A susceptibility test was used to evaluate the antibacterial activities of the cyano-metabolite and its parent FZ. Standard strain of *Escherichia coli* (CCRC 51731) and *Vibrio anguillarum* (CCRC 12908) were obtained from the Food Industry Research and Development Institute (Hsinchu, Taiwan). In the dilution susceptibility test, 100 μl

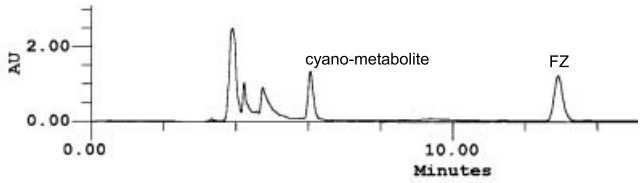


Figure 2.

Mueller–Hinton broth was dispensed in a 96-microwell plate containing a series of drug concentration in the range 0.01–10 $\mu g\,ml^{-1}$, and inoculated with 5×10^6 CFU ml^{-1} of bacteria. Each concentration had three replicates. The lowest concentration of test compound resulting in no growth after 24 h of incubation at 28°C was recorded as the minimum inhibitory concentration.

Results

Isolation and identification of cyano-metabolite

The cyano-metabolite of FZ was biotransformed *in vitro* from FZ using grouper hepatic supernatant. Fractions of cyano-metabolite were identified by its chromatographic behaviour, a retention time about 6 min (figure 2) in the HPLC system, and its ultraviolet spectrum with a λ_{max} at 271 nm using a PDA detector (figure 3a). The yield (table 1) of cyano-metabolite varied under various biotransformation conditions, i.e. aerobic or anaerobic incubation, the concentration of FZ and hepatic supernatant, and the existence of NADP or NADPH cofactors. The optimum condition for cyano-metabolite formation was found when incubating 75 $\mu g\,ml^{-1}$ FZ and 0.3 $g\,ml^{-1}$ of hepatic supernatant in the reaction medium with NADPH as cofactor under an anaerobic condition at 37°C for 1 h. The cyano-metabolite was found to be a major metabolite in this model system.

Pure cyano-metabolite was collected from the fractions of HPLC of similar spectral properties and identified by its nuclear magnetic resonance (NMR) and mass spectra. Electron-impact mass spectrum (EIMS) of cyano-metabolite revealed a low intensity $(M + H)^+$ molecular ion peak at m/z 196, with a base peak at m/z 113. Two major fragments, the oxazolidone and aliphatic nitrile, were detected at m/z 87 and 82, respectively (figure 3b). ^{1}H-NMR spectrum of

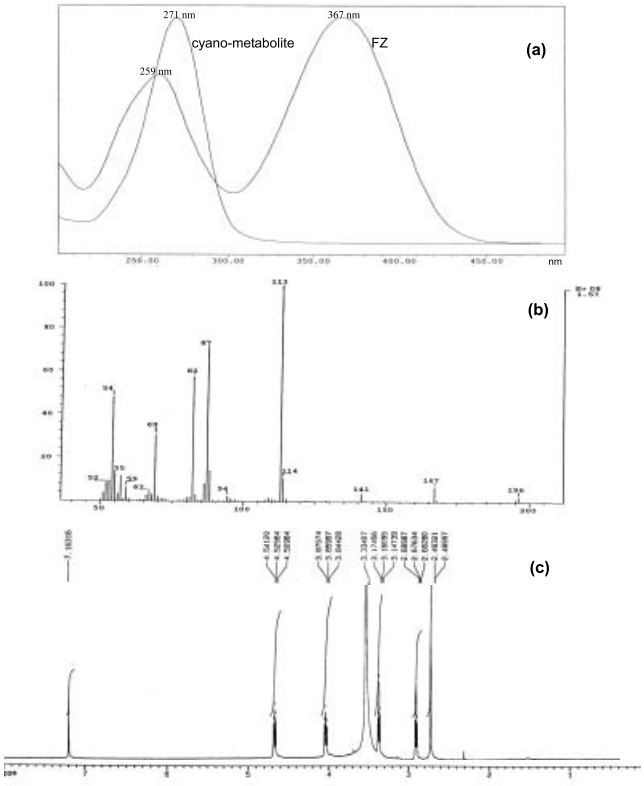


Figure 3.

cyano-metabolite in (dimethyl sulfoxide)-d_6 (figure 3c) showed a singlet at δ7.16 (1H, azomethine proton), two coupled triplets at δ4.53 (2H) and δ3.86 (2H) of methylene protons in the oxazolidone ring, as well as another two coupled triplets at δ3.16 (2H) and δ2.68 (2H) of the vicinal methylene protons in the 4-cyano-2-oxobutylidene moiety which was transformed from the nitro-furan of FZ. Infrared spectrum of cyano-metabolite revealed distinctive absorption bands at 2247 cm^{-1} (C≡N stretching), 1775 cm^{-1} (carbonyl stretching in the oxazolidone ring) and 1703 cm^{-1} (α, β-unsaturated C = O stretching). The white amorphous sample isolated was confirmed as 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone, a cyano-metabolite of FZ.

Dispositions of cyano-metabolite in vivo

In order to observe the disposition of the cyano-metabolite of FZ *in vivo*, various amounts of 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone were injected into the established HPLC for the calibration

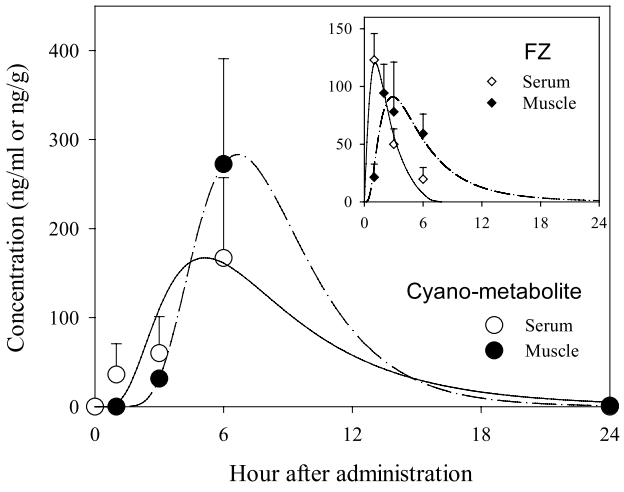


Figure 4.

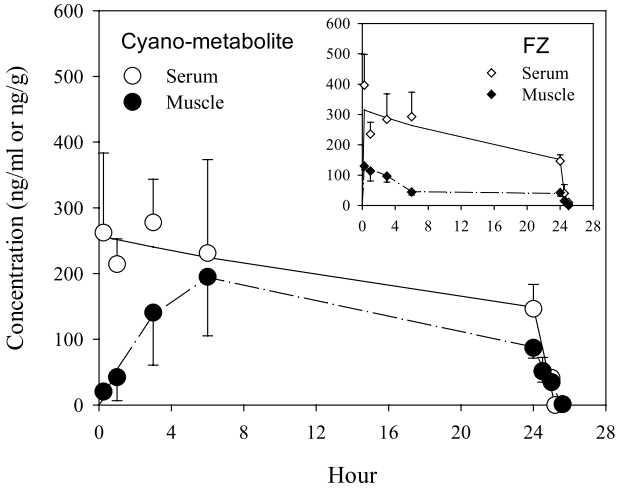


Figure 5.

curve. It was found that the HPLC response of cyano-metabolite was linear over the range 0.1–10 μg ml^{-1} ($r = 0.9999$). A sample preparation procedure was also tested. The mean recoveries of the cyano-metabolite through this sample preparation determined by spiking control tissues with cyano-metabolite were 99.8 ± 4.1% for serum, 98.6 ± 3.5% for muscle, 53.1 ± 7.4% for liver and 64.0 ± 11.4% for kidney. Hence, in this experiment the detection limits of cyano-metabolite in serum, muscle, liver and kidney were 25 ng ml^{-1} and 10, 10 and 25 ng g^{-1}, respectively.

The dispositions of the cyano-metabolite in the orange-spotted grouper treated orally and in bath with FZ are shown in figures 4 and 5 and in table 2. The cyano-metabolite of FZ was found mainly distributed

Table 2. Disposition of 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone, a cyano-metabolite of furazolidone (FZ), in the orange-spotted grouper after oral and bath administration of FZ.

	Oral		Bath	
Parameters*	Serum	Muscle	Serum	Muscle
T_{max} (h)	5.1	6.7	0.25	6
C_{max} (ng ml^{-1} or ng g^{-1})	167.2	283.2	258	204
$t_{1/2e}$ (h)	4.0		0.5	
$T_{withdraw}$ (h)	13.7	16.5	0.9	2.2

*T_{max}, time after administration at which maximum drug concentration was observed; C_{max}, maximum drug concentration; $t_{1/2e}$, the elimination half-life; $T_{withdraw}$, withdrawal period.

in serum and muscle rather than in liver and kidney. After oral treatment of FZ, the peak cyano-metabolite concentration, 167.2 ng ml^{-1} in serum and 283.2 ng g^{-1} in muscle, was reached at 5.1 and 6.7 h, respectively. The elimination half-life of cyano-metabolite measured from the terminal phase of the serum curve was 4 h. Withdrawal periods of cyano-metabolite in serum and muscle were 13.7 and 16.5 h after administration, respectively. The appearance of cyano-metabolite significantly followed the parent FZ and both peak concentrations of cyano-metabolite in serum and muscle were higher than FZ (figure 4 and table 2).

During 24-h bath period of FZ, the peak concentrations of cyano-metabolite, 258 ng ml^{-1} in serum and 204 ng g^{-1} in muscle, were obtained at 0.25 and 6 h, respectively. The half-life of cyano-metabolite was 0.5 h and was not detectable at 0.9 and 2.2 h in serum and muscle, respectively, after transferring the fish to fresh seawater. The distribution of cyano-metabolite in serum was as fast as its parent FZ, but its maximum concentrations in fish tissues were less than FZ (figure 5 and table 2).

Antibacterial activity

The minimum inhibitory concentrations of FZ against *E. coli* and *V. anguillarum* were 0.025 and 0.4 μg ml^{-1}, respectively, but cyano-metabolite showed no antibacterial activity against both bacteria. The results of susceptibility test on *E. coli* and *V. anguillarum* revealed that the cyano-metabolite has no contribution to the antibacterial activity of FZ.

Discussion

Although a variety of pathways have been described for nitrofuran metabolism, nitroreduction appears to be the most biologically significant of these biotransformations. In the present study, the furan ring-opened metabolite 3-(4-cyano-2-oxobutylidene amino)-2-oxazolidone was biosynthesized from the incubation of FZ in grouper hepatic supernatant. Formation of this cyano-metabolite was affected by several factors during *in vitro* enzyme reaction. Our studies revealed that the formation of the cyano-metabolite was higher in the presence of NADPH and under anaerobic conditions, and was obviously affected by the concentration of grouper liver. The enzymatic reduction of FZ to cyano-metabolite has been known occur either under aerobic or anaerobic conditions with either NADP or NADPH as cofactor. Abraham *et al*. (1984) reported that *E. coli* and rat liver enzymes catalysed reductive metabolism of FZ to cyano-metabolite under either aerobic or anaerobic conditions *in vitro*. They also demonstrated that this NADPH-dependent, oxygen-insensitive nitroreductase activity was localized to the microsomal fraction of rat liver and bacterial cell lysates. Tatsumi *et al*. (1981), on the other hand, reported that the milk xanthine oxidase and rat liver 9000*g* supernatant were capable of reducing FZ to cyano-metabolite under strictly anaerobic conditions. Other metabolites were also reported to include a major one, 2,3-dihydro-3-cyanomethyl - 2 - hydroxyl - 5 - nitro - 1a,2 - di(2-oxazolidin-3-yl)iminomethyl-furo[2,3-b]furan, and a minor metabolite, *N*-(5-amino-2-furfurylidene)-3-amino-2-oxazolidone by the same group. These two metabolites were not found in the grouper hepatic supernatant incubation system in this study.

The distribution and elimination of cyano-metabolite in grouper muscle and serum were as fast as its parent FZ after oral or bath treatments of FZ (Guo *et al*. 2002). The time required to reach the peak concentration of cyano-metabolite was about 4 h longer than FZ (figures 4 and 5 and table 2). Another study found that the peak concentrations of FZ, 120.9 ng ml^{-1} in serum and 93.3 ng g^{-1} in muscle, were reached at 1.1 and 2.0 h, respectively, after oral administration of FZ and with an elimination half-life of 0.8 h (Guo *et al*. 2002). In the present study, peak concentrations of cyano-metabolite in serum and muscle after oral treatment of FZ were significantly higher than its parent FZ, especially in muscle with about a threefold difference. Meanwhile, the cyano-metabolite concentration in muscle was also twofold higher than FZ during a 24-h bath treatment with FZ. The withdrawal time of cyano-metabolite in grouper tissues was less than 17 h but longer than FZ (9 h), especially in muscle. The cyano-metabolite was an important and major metabolite in this *in vivo* experiment, a fact also true in the *in vitro* biotransformation of FZ in grouper hepatic supernatant. Similar results were also reported in eel after bath treatment with FZ (Nakabeppu and Tatsumi 1984) and in rat after oral dose of FZ (Tatsumi *et al*. 1984). In swine, cyano-metabolite was reported as a minor metabolite of FZ (Vroomen *et al*. 1987a). Results from this study show that cyano-metabolite in orange-spotted grouper was mainly distributed in serum and muscle rather than in liver and kidney. The same distribution characteristics were also observed in FZ after oral and bath treatments (Guo *et al*., 2002).

The cyano-metabolite of FZ was initially determined from the urine of rabbits receiving FZ orally (Tatsumi *et al*. 1978). It was demonstrated that this cyano-metabolite of FZ is easily formed and discharged in the animals. Fast elimination of cyano-metabolite was also observed in adult swine, wherein no cyano-metabolite in serum, muscle, liver and kidney were detectable at 24, 24, 2 and 2 h, respectively, after oral treatment of FZ (Vroomen *et al*. 1987a).

Low levels of FZ and cyano-metabolite in kidney and liver were detected especially in the orally administered fish. It was inferred that FZ and cyano-metabolite were extremely reactive and metabolized to a more polar metabolites by enzymes or conjugated with macromolecules. Covalent binding of FZ with protein and DNA *in vivo* has been reported in piglets (Vroomen *et al*. 1986), while glutathione-dependent covalent binding of FZ to liver microsomal protein *in vitro* has been reported in rat (Vroomen *et al*. 1987c). A mercaptoethanol conjugate of FZ, 3-(4-cyano-3-β-hydroxyethylmercapto-2-oxobutylidene amino)-2-oxazolidone, which was an adduct of the open-chain acrylonitrile derivative of FZ, was formed by liver microsomes of swine via a reductive process of FZ (Vroomen *et al*. 1987b). It was speculated that cyano-metabolite was the reactive intermediate of FZ to the mecaptoethanol derivative and have a short half-life.

Another important fact observed in this study is that the concentration of FZ was not maintained at the same level during the 24-h bath period and the concentration in muscle was lower than that in serum.

Both FZ and cyano-metabolite in serum showed a gradual decline in concentration since the very beginning of 0.25 h after the immersion of fish in 10 ppm drug solution. Cyano-metabolite in serum seemed to maintain the same decay rate as FZ but at a slightly lower concentration than FZ. This phenomena may be explained by the degradation of FZ in the bath solution that caused the gradual loss of serum concentration, or a self-regulating mechanism that prevented the fish from accumulating the free form of the drug by protein binding or enzyme metabolism. A peak concentration of the cyano-metabolite in muscle obtained 6 h after the bath treatment and a higher concentration of the cyano-metabolite than FZ in muscle, representing a result of a translocation of cyano-metabolite from serum and a transformation from muscle FZ. A fast loss of both FZ and cyano-metabolite in either serum or muscle was also observed after transferring the fish from the bath solution to fresh seawater. Compared with the slow loss of tissue FZ in the orally administer fish, the bath treatment seems an easier way of removing the drug residues in the animal body. This result showed different routes of FZ metabolism while the drug was applied orally or by bathing. The mechanism causing the fast loss of drug needs further investigation, however.

In this study, the results of a susceptibility test on *E. coli* and *V. anguillarum* revealed that the cyano-metabolite has no antibacterial activity in contrast to FZ. Vroomen *et al*. (1987a) also demonstrated that the cyano-metabolite was not mutagenic in the *Salmonella*/microsome test with and without metabolic activation. From the above results, it could be concluded that the cyano-metabolite has no contribution to the mutagenic and antibacterial activity of its parent FZ, but is absolutely related to covalent binding of FZ with macromolecules.

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