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Bath administration of the quinoline antibiotic flumequine to brown trout *Salmo trutta* and Atlantic salmon *S. salar*

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ABSTRACT: Administration of flumequine to brown trout *Salmo trutta* and Atlantic salmon *Salmo salar*, using bath treatments, resulted in significant serum levels of the antibiotic. Bath concentrations of 50, 100 and 500 ppm were tested for up to 5 h. Temperature, pH, and calcium hardness of the bath water were all found to influence serum levels achieved, as did the level of the drug in the bath. Following bath treatment, serum levels of flumequine greater than the minimum inhibitory concentrations for most susceptible fish pathogens were maintained for up to 14 d. Flumequine serum levels eliciting a toxic response in treated fish were determined. The efficacy of flumequine bath treatments in the control of furunculosis, caused by *Aeromonas salmonicida*, has been established and its application as both a prophylactic and a chemotherapeutic method for the control of bacterial infections is proposed.

INTRODUCTION

Flumequine (9-fluoro-6,7-dihydro-5-methyl-1-oxo-1H,5H-benzo-quinolizine-2-carboxylic acid) is a member of the halogenated quinoline carboxylic acid group of antibacterial agents with antimicrobial activity against a wide range of Gram-negative bacteria (Edelson et al. 1977, Neuman 1978, Lemeland et al. 1981). A structural analog of nalidixic acid and oxolinic acid, it has been recommended for the treatment of urinary tract infections (Rohlfing et al. 1976, Steer et al. 1981) and enteric infections (Rohlfing et al. 1977) in humans. It has however been primarily utilised in veterinary medicine for treating domestic animals (Benothame 1979, Ziv et al. 1986).

Both flumequine and oxolinic acid have been routinely administered to farmed fish as both prophylactic and chemotherapeutic agents, principally against systemic bacterial infections, especially furunculosis, caused by *Aeromonas salmonicida* (Michel et al. 1980, Austin et al. 1983, Scallan & Smith 1985) and enteric red mouth disease, caused by *Yersinia ruckeri* (Rodgers & Austin 1983). A third quinoline antibiotic, piromidic acid, has been recommended as a safe and effective compound for the treatment of bacterial infections in goldfish *Carassius aurata* and eel *Anguilla japonica* (Katae et al. 1979) and against furunculosis in ayu *Plecoglossus altivelis* (Sano unpubl.).

The widespread use of flumequine for the treatment of bacterial infections in farmed fish is due mainly to its relatively low minimum inhibitory concentration (MIC) for most susceptible fish pathogens (Ledo et al. 1987, O'Grady et al. 1987) and effective systemic distribution when administered orally via medicated food (Chevalier et al. 1981). However, diseased fish tend to be anorexic, and this gives rise to a situation where only clinically healthy individuals within a population (i.e. those that are still feeding) are likely to be protected by the oral administration of antibiotics. This has led to the examination of alternative modes of administration of these antibiotics.

Intraperitoneal injection (i.p.) represents the most effective, though laborious, means of administering an accurate therapeutic dose of flumequine to an individual. However, the stress that this method can impose on diseased fish renders it inappropriate as a method of chemotherapy. Nevertheless, this method has been used successfully for the elimination of *Aeromonas salmonicida* from asymptomatic carriers in populations of Atlantic salmon prior to their transfer from freshwater to seawater (Scallan & Smith 1985).

In an attempt to achieve high systemic flumequine

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levels comparable to those obtained with an i.p. injection while avoiding its attendant stress, we studied the feasibility of administering flumequine by the bath method. Our objective was to develop a prophylactic and chemotherapeutic treatment for the control of bacterial infections of farmed fish.

MATERIALS AND METHODS

Experimental fish. The fish used in these experiments were either brown trout *Salmo trutta* (average weight 28.72 g) or Atlantic salmon *Salmo salar* (average weight 28.43 g). The brown trout were held in 500 l flow-through tanks at ambient temperature (7 to 12 °C) until required. With one exception experimental fish were not fed for 3 wk before use, nor during experiments. The exception was an experiment, described below, to test the effect of feeding on drug absorption. The pre-smolt salmon were transported to this laboratory from a fish hatchery where a clinical outbreak of furunculosis was in its second week. The 500 l flow-through tanks used for holding the fish and the 50 l flow-through tanks to which test fish were removed after flumequine bath administrations were supplied with dechlorinated water (pH 7.1 to 7.8; 132 to 173 mg l^{-1} $CaCO_3$; 78 to 89 mg l^{-1} total alkalinity). Distilled water (pH 6.5 to 6.7; 1 to 2 mg l^{-1} $CaCO_3$; 10 to 12 mg l^{-1} total alkalinity) was used in all bath administrations. At all times both the control and treated groups of fish were maintained in *Aeromonas salmonicida*-free water (Scallan 1983).

Bath administrations. All of the bath administrations carried out in the laboratory were run in 10 l plastic buckets containing 4 l of the antibiotic solution or in 100 l plastic tanks containing various volumes of the antibiotic solution. The antibiotic solution was prepared by diluting flumequine (Flumisol-10 %, Riker Labs., France) in distilled water to the final desired concentration of 50, 100, or 500 ppm. If necessary, the pH was then adjusted to the desired level with 1 *N* NaOH (BDH Chemicals Ltd., Poole, England) for a particular bath treatment. The bath solutions were aerated and were placed in a water bath to attain the desired temperature for each particular bath treatment. Calcium hardness levels were adjusted by the addition of calcium oxide (BDH Chemicals Ltd) to the antibiotic baths. The calcium levels were determined by the standard EDTA titration method and expressed as mg l^{-1} $CaCO_3$ (Franson et al. 1974).

Test fish were held at the same temperature as the bath for 24 h before being used in each laboratory bath treatment (1 fish l^{-1} antibiotic solution). Serum samples from treated fish were obtained by removing blood from the caudal vein following anaesthesia with benzocaine (50 ppm, Sigma Ltd, Poole, England). Serum samples were also taken from untreated fish as controls. The blood was allowed to stand at room temperature for 1 h and was then held overnight at 4 °C to allow clotting to proceed. Blood samples were centrifuged and the serum removed and stored at −20 °C. Bioassays, as described below, were carried out within 1 wk of bleeding.

The flumequine assay used was a standard agar cup-plate diffusion assay following the guidelines of Reeves & Bywater (1976). A tryptone soya broth (Oxoid Ltd, Basingstoke, England) culture of *Vibrio anguillarium* 775, with 1 % added NaCl and an optical density of 0.1 at 540 nm, was used (5 ml) to seed 200 ml of tryptone soya agar (TSA, Oxoid) plus 1 % NaCl. Five doubling dilutions of a flumequine stock solution ranging from either 48 to 3 μg ml^{-1}, 32 to 2 μg ml^{-1}, or 16 to 1 μg ml^{-1} were used in each plate. Test and control fish sera were added to 1, 2, or 3 wells. A second flumequine stock solution was used to prepare 2 control concentrations. The plates were incubated at 30 °C overnight and zones of inhibition of growth around the wells were measured. For all assays, a standard curve was computed for each test plate by regression analysis. The concentration of flumequine in the test sera was calculated and corrected for error by reference to the controls. A correlation coefficient was calculated for each curve and all were shown to be highly significant at the 99 % confidence level ($r = 0.998$, $p < 0.001$).

Retention times of flumequine serum levels. Fifty-two brown trout were given 50 ppm flumequine bath treatment at 11 °C for 3 h in distilled water. Four fish were removed at 0 h, and 4 more at 1 h and at 3 h during the bath treatment. Blood samples were taken and flumequine serum levels determined.

At the termination of the bath treatment the remaining 40 fish were removed to a 50 l flow-through tank at ambient temperature (7 to 10 °C) for 14 d. Four fish were removed at each of the following times post-treatment: 1 h, 3 h, 5 h, 8 h, 12 h, 1 d, 3 d, 6 d, 10 d, and 14 d. Blood samples were taken from these fish and flumequine serum levels determined.

Forty brown trout were anesthetised with benzocaine (50 ppm) and injected i.p. with a 3 % injectable flumequine suspension (Flumiquil, Riker Labs.) diluted in 0.85 % saline to give a dosage of 30 mg kg^{-1} fish. The fish were then maintained and serum levels determined in the same way as for bath-treated fish.

Effect of feeding on flumequine absorption. Two groups of 20 brown trout were held in 500 l flow-through tanks at ambient temperature (11 to 12 °C) for 21 d. One group was fed at a rate of 1 % body weight per day whilst the second group received no feed. On the 21st day both groups were placed in a flumequine bath solution of 50 ppm, at 11 °C and pH 7, in distilled

water for 1 h. The 2 groups were kept apart by means of a perforated screen. At the termination of the bath treatment, blood samples were taken from 10 fish of each group and flumequine serum levels determined.

Toxicity of bath-administered flumequine. Forty brown trout were given a flumequine bath treatment of 50 ppm at 11 °C and pH 6.5 (40 l). At the first sign of toxicity (onset of lethargy) 10 fish were sampled to determine flumequine serum levels. At the same time 10 fish were removed to a 50 l flow-through tank at ambient temperature (12 °C) where their recovery was monitored. When the remaining 20 fish in the flumequine bath lost their equilibrium, 10 fish were sampled to determine flumequine serum levels and the remaining 10 were removed to a 50 l flow-through tank where their recovery was monitored.

Bath treatment of salmon suffering from furunculosis. Three hundred pre-smolt Atlantic salmon from a population undergoing a clinical outbreak of furunculosis were transported to this laboratory from a local fish hatchery and maintained in a 500 l flow-through tank at ambient temperature (12 °C). Two d later, 200 fish received a flumequine bath treatment of 50 ppm for 3 h at 11 °C and pH 7 in distilled water (100 l). Immediately after treatment, 4 fish were bled to determine the flumequine serum levels achieved. The remainder of the group were returned to a 500 l flow-through tank at ambient temperature (12.5 °C). Two d post-treatment the fish were stressed by an intramuscular injection of prednisolone acetate (PA, Boots PLC, Nottingham, England) at a dosage of 20 mg kg^{-1} fish. The stressed fish were than held in a 150 l tank at 18 °C for 14 d. The remaining 100 fish were used as an untreated control group. The control fish were stressed in the same manner and at the same time as the treated fish.

Kidney material from dead fish was streaked on TSA and incubated at 22 °C for 48 h. On isolation of colonies producing a brown diffusable pigment within 48 h, mortalities were attributed to *Aeromonas salmonicida*. At the termination of the stress, test kidney material of the surviving fish from both the treated and the control groups was tested for the presence of *A. salmonicida* as described above. The sensitivity of the *A. salmonicida* isolates to both flumequine and oxolinic acid was tested by the standard Kirby-Bauer method (Brown & Blowers 1978).

RESULTS

Effect of flumequine concentration on absorption

Serum levels achieved in brown trout by a bath administration of flumequine at various concentrations at 11 °C and pH 7 are presented in Fig. 1. At a concentration of 500 ppm, high serum flumequine levels were

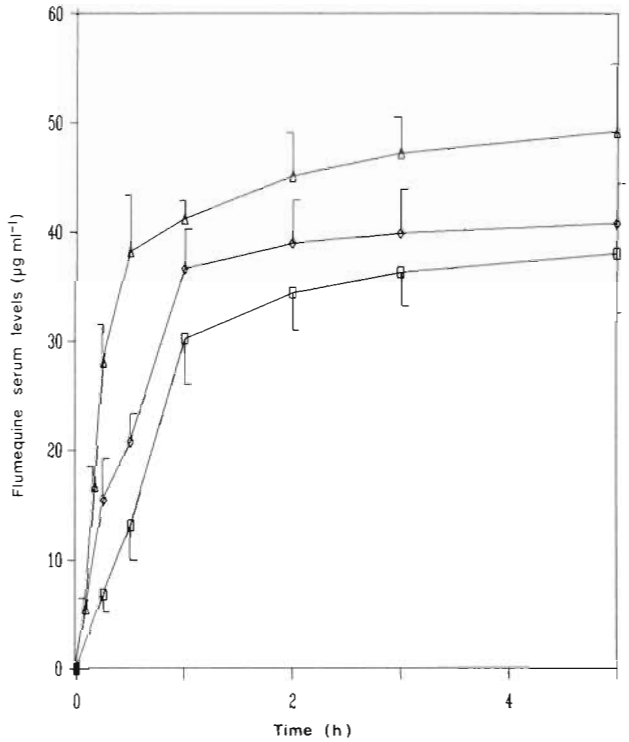

Fig. 1. *Salmo trutta*. Effect of concentration and duration of treatment of flumequine absorption. Distilled water at 11 °C and pH 7.0 with (□) 50 ppm flumequine; (◊) 100 ppm flumequine; (△) 500 ppm flumequine

rapidly achieved. The rate of absorption was greatest in the first 30 min, with serum levels of 38 µg ml^{-1} (± 5.2) being attained. Subsequently, the rate of absorption declined, with serum levels reaching 49 µg ml^{-1} (± 6.1) after 5 h of treatment. Administration of flumequine at 50 and 100 ppm resulted in similar patterns of drug absorption. In the 100 ppm bath, the initial rate of absorption was rapid, with serum levels of 37 µg ml^{-1} (± 3.7) being achieved after 1 h of treatment. The rate of absorption subsequently decreased and levels of 41 µg ml^{-1} (± 3.8) were present after 5 h of treatment. The 50 ppm bath treatment resulted in serum levels of 30 µg ml^{-1} (± 4.2) and 38 µg ml^{-1} (± 5.4) following 1 and 5 h of treatment, respectively.

Bath administration of flumequine at 50 and 100 ppm for 1 h in distilled water achieved high serum levels with no apparent toxic effects. These baths were therefore used as the standard tests for studying the effect of various parameters on flumequine absorption.

Effect of pH on absorption

The effect of pH on the absorption of flumequine was studied using 50 and 100 ppm baths for 1 h at 11 °C. Results are presented in Fig. 2. A progressive decline in

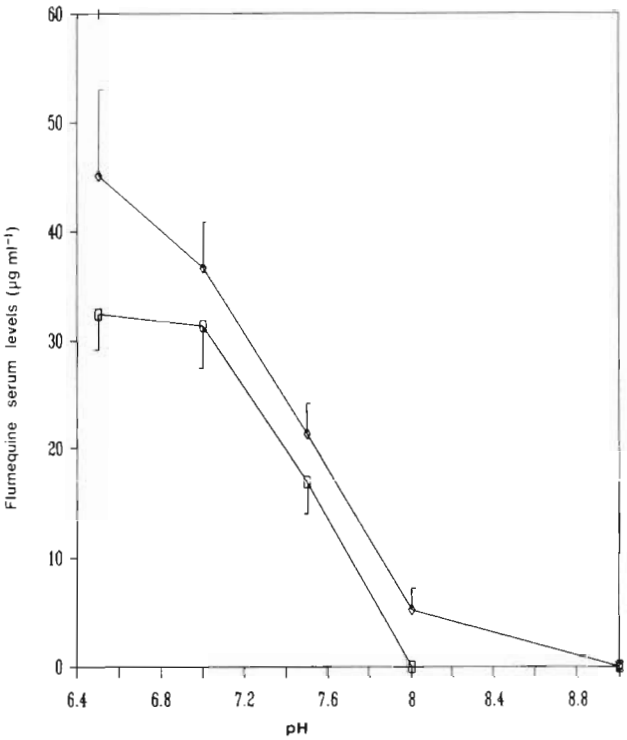

Fig. 2 *Salmo trutta*. Effect of pH on the absorption of bath-administered flumequine. Distilled water at 11 °C with (□) 50 ppm flumequine; (◊) 100 ppm flumequine

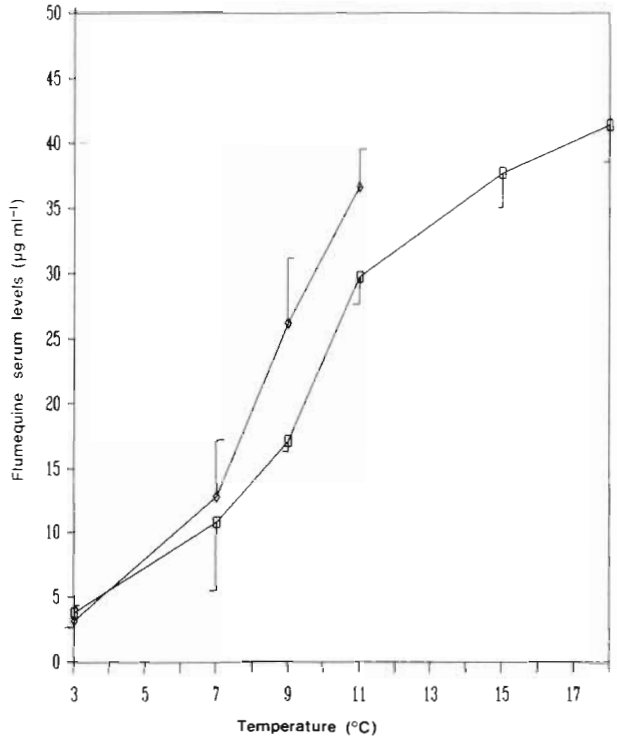

Fig. 3. *Salmo trutta*. Effect of temperature on the absorption of bath-administered flumequine. Distilled water at pH 7.0 with (□) 50 ppm flumequine; (◊) 100 ppm flumequine

uptake occurred with increasing pH, with observed levels falling from 35 µg ml^{-1} (± 4.3) at pH 7 to 5 µg ml^{-1} (± 2.0) at pH 8 for the 100 ppm bath test. No detectable serum levels were observed at pH 9. A similar result was observed with the 50 ppm bath test where flumequine serum levels fell from 31 µg ml^{-1} (± 3.9) at pH 7 to undetectable levels at pH 8. With both the 50 and the 100 ppm bath treatments at pH 6.5, flumequine was observed to crystallise out of solution due to its low solubility at acidic pH. A slight toxic effect was observed at pH 6.5 during the 100 ppm treatment where the 4 treated fish appeared lethargic compared to their counterparts in the 50 ppm treatment.

Effect of temperature on absorption

The effect of temperature on the absorption of flumequine was studied using 50 and 100 ppm baths for 1 h at pH 7. Results are shown in Fig. 3. In the 50 ppm bath treatment the relationship between serum levels of flumequine and temperature is approximately linear between 3 and 15 °C with levels of 4 µg ml^{-1} (± 1.2) and 38 µg ml^{-1} (± 2.6) being recorded at these temperatures, respectively. A levelling off in serum levels is seen at 18 °C (41 µg ml^{-1}, ± 2.8). The 100 ppm bath treatment showed a similar linearity in the absorption-temperature relationship between 3 °C, (3 µg ml^{-1}, ± 1.1), and 11 °C, (38 µg ml^{-1}, ± 2.9). However, the levels of absorption were not determined at 15 and 18 °C.

Effect of calcium hardness on absorption

The effect of various calcium hardness levels (as mg l^{-1} $CaCO_3$) on the absorption of flumequine was studied using 50 ppm baths for 1 h at pH 7 and 11 °C. Results are presented in Fig. 4. Flumequine serum levels of 29 µg ml^{-1} (± 2.1) were achieved by the standard bath test in the absence of added calcium. A decrease in flumequine absorption with increasing levels of calcium was most pronounced at calcium levels up to 75 mg l^{-1} $CaCO_3$. At higher calcium levels, the rate of decrease in flumequine absorption was less pronounced. At 75 mg l^{-1} $CaCO_3$ serum levels had decreased to 16 µg ml^{-1} (± 4.0) and thereafter decreased to 10 µg ml^{-1} (± 2.0) at 200 mg l^{-1} $CaCO_3$.

Retention times of flumequine serum levels

Retention times of bath-administered flumequine in the serum following 3 h of immersion in 50 ppm of the drug at pH 7 and 11 °C in distilled water, and following

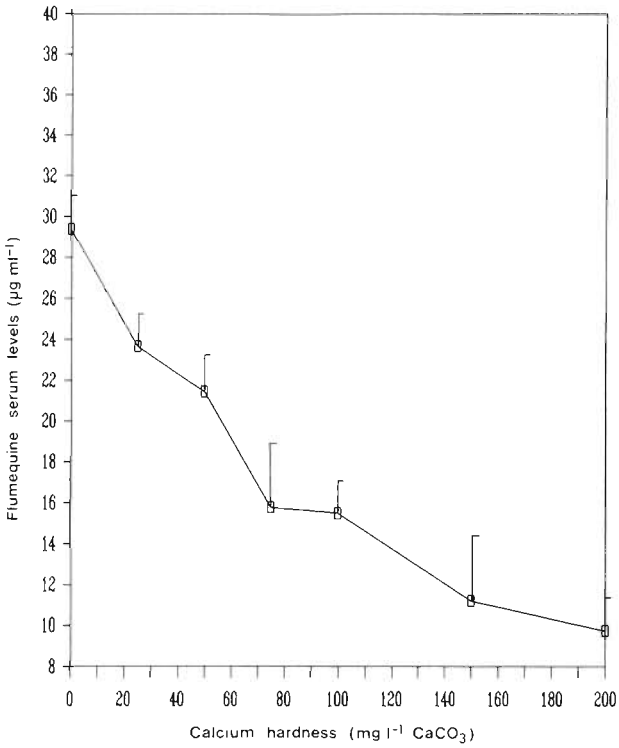

Fig. 4. *Salmo trutta*. Effect of calcium hardness on the absorption of bath-administered flumequine. Distilled water at 11 °C and pH 7.0 containing 50 ppm flumequine and various concentrations of calcium

an i.p. injection, are presented in Figs. 5a and b. Bath administration of flumequine resulted in peak serum levels of 43 µg ml^{-1} (± 11.8 which fell to 21 µg ml^{-1} (± 3.1) after 24 h and 11 µg ml^{-1} (± 5.2 after 3 d). Subsequently, the rate of flumequine elimination decreased with serum levels of 1 µg ml^{-1} (± 0.4) still being present 14 d after treatment.

The curve depicting serum retention time following an i.p. injection of 30 mg flumequine (kg fish)$^{-1}$ showed a similar result to that reported by Scallan & Smith (1985). Peak serum levels of 31 µg ml^{-1} (± 3.6) were observed 3 h after treatment, after which they dropped to 15 µg ml^{-1} (± 2.3) by 24 h post-treatment. Subsequent elimination of injected flumequine (Fig. 5b) proceeded at a slower rate than occurred with the bath-administered drug, with serum levels of 12 µg ml^{-1} (± 2.2) and 3 µg ml^{-1} (± 1.4) being recorded after 3 and 14 d respectively.

Effect of feeding on absorption

The bath treatment of both fed and unfed brown trout in the same bath solution resulted in similar flumequine serum levels being recorded for both groups. Serum levels of 36 µg ml^{-1} (± 9.3) were

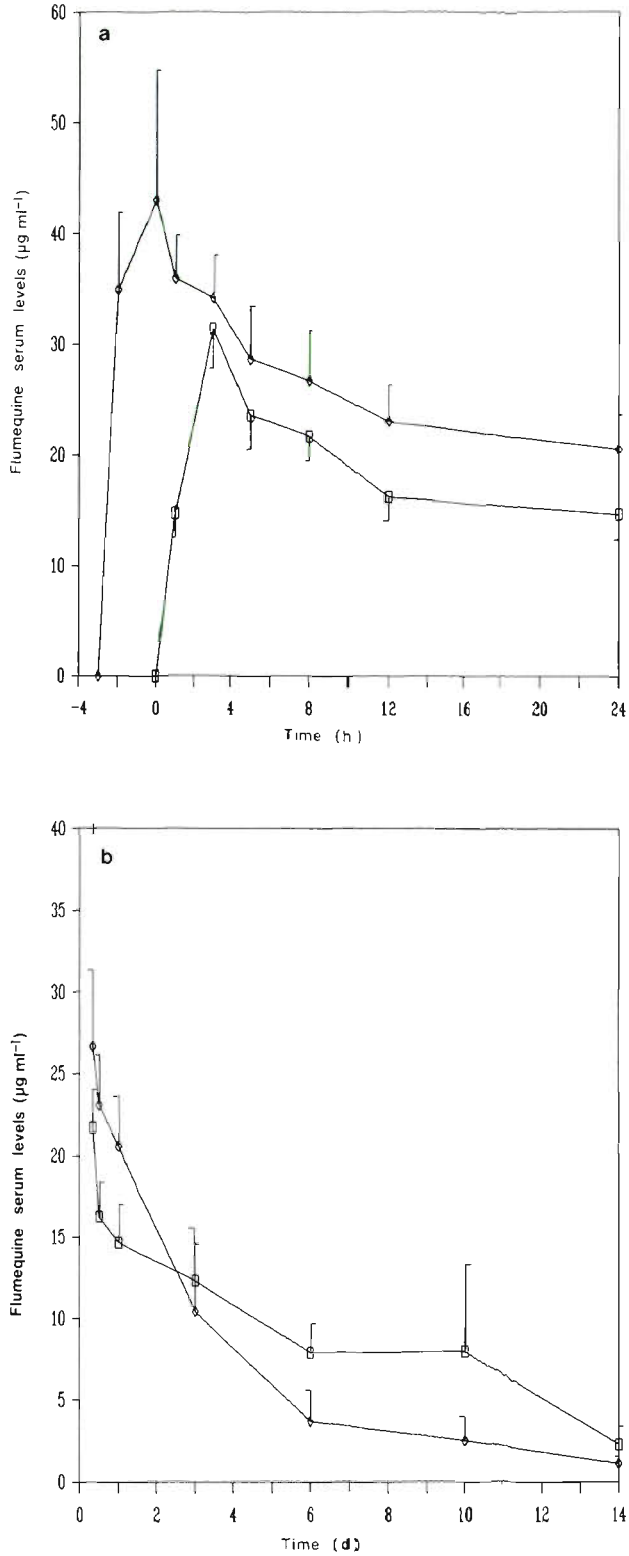

Fig. 5. *Salmo trutta*. Retention times of flumequine serum following administration of the drug by bathing and injection. (◊) Bath administration of flumequine at 50 ppm, 11 °C and pH 7.0 in distilled water for 3 h; (□) intraperitoneal injection of flumequine at 30 mg kg^{-1} fish. (a) First 24 h after treatment; (b) first 14 d after treatment

observed in fish that had been fed at 1 % body weight per day for 3 wk. The group that received no feed showed serum levels of 33 μg ml^{-1} (± 4.2).

Toxicity of bath-administered flumequine

The first sign of flumequine toxicity, namely lethargy in the treated fish, became apparent after 1 h of treatment in a bath containing 50 ppm of the drug at pH 6.5 and 11°C. At this stage, serum levels of 36 μg ml^{-1} (± 9.3) were observed. All 10 fish removed from the bath at this point recovered within 1 h. The remaining fish lost their equilibrium after 4.5 h of treatment. Flumequine serum levels of 69 μg ml^{-1} (± 12.6) were recorded at this point and 9 of the 10 remaining fish regained their equilibrium within 1 h of removal from the bath. The remaining fish died.

Bath treatment of salmon suffering from furunculosis

The administration of a 50 ppm flumequine bath treatment at pH 7 and 11 °C to Atlantic salmon pre-smolts undergoing a clinical outbreak of furunculosis yielded serum levels of 34 μg ml^{-1} (± 8.8) in the treated group of 200 fish. No mortalities were observed in this group in the 14 d following treatment. In the untreated control group of 100 pre-smolts, mortalities of 32 %, attributable to *Aeromonas salmonicida*, were observed.

In post-mortem examinations carried out on the surviving fish at 14 d post-treatment. *Aeromonas salmonicida* was isolated from 1 individual in the treated group and from 15 individuals in the untreated group. All of the *A. salmonicida* isolates tested showed sensitivity to both flumequine and oxolonic acid.

DISCUSSION

Bath treatments have found their greatest use in the topical administration of chemicals such as formalin and malachite green to farmed fish (Herwig 1979). However, the application of antibiotics using baths has also been proposed for furanace (Shimizu & Takase 1967), erythromycin (Swarz 1982), oxytetracycline (Herwig 1979), nifurprazine (Herwig 1979), and oxolinic acid (Endo et al. 1973).

The absorption of bath-administered flumequine followed a definite pattern irrespective of flumequine concentration (Fig. 1). This was manifest in the rapid rate of drug absorption in the initial stages of the treatment (0.5 to 1 h) and in the subsequent marked decrease in the rate of drug absorption (1 to 5 h).

The pH of the bath solution exerted a strong influence on the uptake of bath-administered flumequine. In general, as the pH of the bath solution decreased the amount of flumequine absorbed increased. Temperature also significantly influenced the uptake of flumequine, with good absorption being observed at temperatures above 7 °C. Fortunately such temperatures are most likely to be encountered in field situations during periods when treatments are necessary. Calcium has been reported to bind flumequine in aqueous solutions (Robert Collas, Riker Labs., pers. comm.). This property of calcium may explain the results in Fig. 4 which show reduced absorption of flumequine in the presence of increasing levels of calcium. Considering the effect of temperature, pH, and calcium hardness, good results can be achieved with a 50 ppm bath from 7 to 15 °C, and pH 6.5 to 7.5 utilising a natural water source containing low levels of calcium hardness (ca 50 mg l^{-1}).

As standard oral antibiotic therapy is normally applied for 7 to 10 d it was felt that flumequine bath administrations must be able to achieve and maintain serum levels greater than the MIC for most susceptible fish pathogens for 10d. Ledo et al. (1987) found the MIC of flumequine against *Aeromonas salmonicida* to be <0.075 μm ml^{-1}, against *Vibrio anguillarium* to be <0.075 to 0.3 μg ml^{-1}, and against *Yersinia ruckeri* to be 0.075 to 0.3 μg ml^{-1}. Fig. 5a and b demonstrate that a flumequine bath administration, which achieved peak serum levels of 43 μg ml^{-1} (± 11.8), maintained serum levels in excess of these MIC values for at least 14d.

The toxic side effects observed in treated fish fell into 2 categories. In one, the treated fish became lethargic; the lethargy occurred at flumequine serum levels of ca 40 μg ml^{-1}. In the other, treated fish lost their equilibrium; this reaction occurred at serum levels of ca 70 μg ml^{-1}

Preliminary field trials with flumequine baths using a natural water source and feeding fish have consistently achieved serum levels in excess of those observed in the laboratory using distilled water (unpubl.). Considering the data resulting from the fed/unfed experiment, it is unlikely that this difference in absorption is a consequence of the fact that the fish in the field trials were feeding. The possibility therefore exists that distilled water has an adverse effect on absorption or that there are other undefined parameters which influence flumequine absorption when natural waterbodies are used for a bath administration.

The efficacy of the bath treatment in preventing mortalities in a stressed group of experimental fish suggests that this method should provide a viable chemotherapeutic method for the administration of flumequine to farmed fish. The achievement of flumequine serum levels in excess of those achieved by the recommended i.p. dose of 30 mg kg^{-1} fish (Scallan &

Smith 1985) suggests that bath administrations of flumequine may also find application as a prophylactic method for the control of furunculosis in farmed salmon prior to their transfer to seawater. However, the environmental considerations involved in adding antibiotics to farm waters during bath treatments must also be addressed. Because of this, bath treatments may be of limited use until a safe disposal method for the residual antibiotics in baths is developed.

The future use of flumequine will also depend on its efficacy in combating pathogenic bacteria resistant to the quinoline antibiotics. The isolation of strains of *Aeromonas salmonicida* possessing 2 separate levels of resistance to flumequine and oxolinic acid has been reported (O'Grady et al. 1987). Furunculosis caused by these pathogenic strains was reported to be unaffected by oral oxolinic acid therapy at 3 times the recommended dosage (O'Grady et al. 1987). Research on the efficacy of flumequine bath administrations in controlling epizootics caused by the oxolinic acid resistant strains is in progress.

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