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Pharmacokinetics of florfenicol in cod *Gadus morhua* and *in vitro* antibacterial activity against *Vibrio anguillarum*

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ABSTRACT: The pharmacokinetic profile of the antibacterial agent florfenicol was studied in plasma after intravenous (i.v.) injection and in plasma, muscle and liver following oral (p.o.) administration to cod *Gadus morhua*, held in seawater at 8°C and weighing 100 to 200 g. Following i.v. injection, the plasma drug concentration-time profile showed 2 distinct phases. The plasma distribution half-life ($t_{1/2}\alpha$) was estimated to be 1.6 h, the elimination half-life ($t_{1/2}\beta$) to be 43 h, the total body clearance (Cl_T) to be 0.015 l kg^{-1} h^{-1} and mean residence time (MRT) to be 74 h. The volume of distribution at steady state, $V_{d(ss)}$, was calculated to be 1.1 l kg^{-1}. Following p.o. administration, the bioavailability was estimated to be 91%, the peak plasma concentrations (C_{max}) to be 10.8 µg ml^{-1} and the time to peak plasma concentrations (T_{max}) to be 7 h. Corresponding C_{max} and T_{max} values were 13.0 µg g^{-1} and 9 h, respectively, in muscle and 12.1 µg g^{-1} and 9 h, respectively, in liver. The *in vitro* minimum inhibitory concentration (MIC) values of florfenicol against 3 *Vibrio anguillarum* strains isolated from diseased cod (A-21, HI-610, HI-618) were 0.5 µg ml^{-1} for all 3 strains.

KEY WORDS: Fish · Pharmacokinetics · Florfenicol

INTRODUCTION

The cod *Gadus morhua* is regarded as an interesting candidate to domesticate for aquaculture purposes and during the last several years large-scale production of cod fry has been established, which will presumably lead to an increase in the output of fish for consumption (Svåsand & Taranger 1998, Kvenseth & Borthen 2002). However, bacterial infections, especially due to *Vibrio anguillarum*, cause heavy losses in the production of cod fry, with outbreaks usually occurring prior to or in connection with vaccination (Bergh 2002). Clinical infections are typically associated with low mortality over a long period (Torrissen et al. 1993). When outbreak occurs, antimicrobial therapy is essential in order to treat the infection. To establish correct dosage regimes and thereby promote optimal use of a drug, data derived from pharmacokinetic investigations and knowledge of the pathogen's susceptibility to the drug in use is essential. Since the pharmacokinetic properties of antibacterial agents vary between species, drugs should be investigated in the species in which they are intended to be used (Kleinow et al. 1994, Martinsen et al. 1994, van der Heiden et al. 1994). In cod, available pharmacokinetic data is limited to studies by Hansen & Horsberg (2000) and Samuelsen et al. (2003), where the pharmacokinetic properties of the quinolones flumequine and oxolinic acid, respectively, were examined following intravenous injection (i.v.) and oral (p.o.) administration. In a study by Bergsjø (1974), sulphadimidine was administered to cod by bathing.

Florfenicol is at the moment the second most used antibacterial agent in Norwegian aquaculture (Grave et al. 1999). The drug possesses good activity against fish pathogens and the bioavailability of florfenicol in Atlantic salmon *Salmo salar*, at 99%, is excellent (Horsberg et al. 1996). However, medication with a daily dose of 10 mg kg^{-1} of florfenicol did not have the expected efficacy in treating vibriosis in cod fry in a

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field situation (J. P. Pedersen pers. comm.). After 5 d of treatment daily mortality decreased only slowly and, for this reason, florfenicol was replaced by flumequine, with good results. The *in vitro* minimum inhibitory concentration (MIC) value for the presumed pathogen against florfenicol was found to be 0.5 µg ml^{-1}. This study was therefore initiated to examine the pharmacokinetic properties of florfenicol in cod, relate the data to the MIC values of bacterial strains isolated from diseased cod and other fish pathogenic bacteria, and evaluate the need for adjustment of the dosage regime. The drug was administered by i.v. injection and incorporated in feed for p.o. administration.

MATERIALS AND METHODS

Chemicals. The Directorate of Fisheries, Department of Quality Control (Bergen, Norway) supplied florfenicol and florfenicol amine standards. Thiamphenicol was obtained from Sigma Chemical and metomidate from Norsk Medisinaldepot. 1-Heptane sulphonic acid was from Fluka Chemie, whereas acetonitrile, methanol (high performance liquid chromatography [HPLC]-grade), phosphoric acid (H_3PO_4), disodium hydrogen phosphate (Na_2HPO_4), trisodiumphosphate (Na_3PO_4), 5-sulphosalicylic acid, hydrochloric acid (HCl), tris(hydroxymethyl)-aminomethan (TRIS), triethylamine and potassium hydroxide (KOH) (p.a.-grade) were all from Merck. Solid phase extraction columns (SPE) (Bond Elute C-18, 100 mg, 1 ml) were from Varian. Stock solutions of florfenicol, florfenicol amine and thiamphenicol were prepared at a concentration of 0.1 mg ml^{-1} in methanol and stored at –20°C. Working standards were prepared by dilution from the stock solutions with methanol.

Antimicrobial formulations. The medicated feed for the p.o. administration was made by homogeneously mixing 4 g Aquaflor Vet. premix (50%) from Schering-Plough with 100 g of ground pellets (Skretting) followed by addition of water (100 ml) to a final constituent of moist feed and a concentration of 10 mg florfenicol per g feed. For the i.v. injection, florfenicol was dissolved in propylene glycol:saline (50:50%) to a final concentration of 10 mg ml^{-1}.

Experimental fish. Cod from Parisvannet Research Station (Institute of Marine Research, Norway) varying in weight from 100 to 200 g were kept in a flow-through storage tank (1 × 2.5 m internal diameter) at the Institute of Marine Research (Bergen, Norway). The seawater had a salinity of 33‰, a temperature of 8.0 ± 0.5°C and a flow-rate of approximately 12 l min^{-1}. Following an acclimatisation period of 3 wk, fish from the storage tank were randomly divided into 2 groups, each of 60 individuals, and maintained in circular (0.8 × 0.8 m internal diameter) flow-through seawater tanks with a flow rate of 8 l min^{-1}. The fish were starved for 2 d prior to drug administration and were not fed the first 4 d in the experimental period.

Drug administration. Prior to drug administration, the fish were lightly sedated by immersion in a solution of metomidate (5 mg l^{-1}) in seawater for approximately 3 min and weighed.

To the fish in Group 1, the solution containing florfenicol was slowly injected into the caudal vein using a 1 ml disposable syringe. The injection volume was 1 ml kg^{-1} body weight, corresponding to a dose of 10 mg kg^{-1} $fish^{-1}$. The position of the needle in the caudal vein was confirmed by aspirating blood into the syringe prior to and after injection. If the needle had translocated during injection or the fish was heavily bleeding after redrawal of the needle, the fish was excluded from the study and replaced.

To the fish in the Group 2, medicated feed was administered into the stomach using a silicon hose and a piston. The amount of feed was 1 g kg^{-1} $fish^{-1}$, corresponding to a dose of florfenicol of 10 mg kg^{-1} $fish^{-1}$. Following administration, each fish was observed for possible regurgitation over a period of approximately 5 min. Fish that regurgitated feed were excluded from the study and replaced.

Sampling. At every sampling point, 6 fish from each group were killed by a blow to the head and samples of plasma, muscle and liver were obtained. Blood was sampled from the caudal vein using a heparinized 1 ml syringe and collected at least 2 cm away from the injection site in the i.v.-injected fish. Plasma was isolated by centrifugation of the blood at approximately 800 g for 10 min. All samples were immediately frozen and stored at –20°C until analysed. For the i.v.-injected group (Group 1) the first samples were taken after 1 h and then at periodic intervals for 168 h. For the p.o. administered group (Group 2) the first samples were taken 2 h following administration and then at periodic intervals for 168 h.

Sample preparation. The plasma and tissue samples were prepared and analysed following a modified procedure of previously published methods (Hormazabal et al. 1993, Vue et al. 2002). To 250 µl fish plasma was added 100 µl distilled water, 250 µl of a 0.3 M solution of sulfosalisylic acid and 10 µl of a methanol solution containing thiamphenicol (100 µg ml^{-1}) as internal standard. After shaking, the samples were centrifuged at 12 000 × *g* in a Biofuge A Bench-centrifuge (Heraeus Sepatech) for 5 min. Following centrifugation, the supernatant was added to 130 µl of a 1 M KOH solution and 500 µl of a 0.1 M TRIS/HCl buffer (pH 8.2) and applied to a solid phase extraction (SPE) column, pre-conditioned successively with 1 ml of methanol and 1 ml of distilled water. The column was washed with 500 µl of

distilled water:acetonitrile (85:15%) and dried prior to elution of the drugs with 500 µl of distilled water:acetonitrile (50:50%). The vacuum pump used was the model E2M 1.5 from Edwards High Vacuum. Twenty µl of the elute was injected into the HPLC system.

Prior to homogenisation of muscle and liver samples (1.0 g), 40 µl of a 100 µg ml^{-1} solution of thiamphenicol in methanol and 1 ml of a solution of 0.9% NaCl in water were added. Following homogenisation, 1 ml acetone was added to the homogenate, shaken and centrifuged for 5 min at 2500 *g*. The supernatant was extracted with 2 ml methylene chloride followed by evaporation of the methylene chloride fraction to dryness using nitrogen. The residue was dissolved in 500 µl of a solution of 0.01 M Na_2HPO_4 (pH 2.8): methanol (80:20%), washed with 1 ml hexane and filtered through a Spin-X Micro Centrifuge Filter (0.2 µm) from Corning. Twenty µl of the filtrate was used for the HPLC analysis.

Plasma, muscle and liver samples from 3 fish were taken prior to initiation of the study and analysed to confirm the absence of florfenicol, florfenicol amine and thiamphenicol. Standard curves for florfenicol and florfenicol amine in the range of 0.1 to 10.0 µg g^{-1} (ml^{-1}) using an internal standard concentration of 4.0 µg g^{-1} (ml^{-1}) were prepared in triplicate.

Analytical procedure. The HPLC system used consisted of an SP 8800 ternary HPLC-pump (Spectra-Physics) connected to a Gilson 234 Autoinjector (Gilson) and a Spectra-Physics SP-8480 UV-detector operating at a wavelength of 225 nm. The integrator was the model SP-4270 from Spectra-Physics. The analytical column was a 150 × 4.6 mm Zorbax SB-C-18, 3.5 µm (Agilent Technologies) connected to a short C-18 pre-column (10 × 4.6 mm). The column was operated at room temperature. The mobile phase was a mixture of 2 solutions, A and B, at a ratio of 68:32%. Solution A was 0.02 M heptansulphonate and 0.025 M Na_3PO_4 in water regulating the pH to 3.85 using phosphoric acid (1 and 5 M). Solution B was methanol containing 0.1% triethylamine. The mobile phase was filtered through a 0.2 µm Millipore filter and degassed using helium and sonication (5 min). The flow rate was 1 ml min^{-1}, giving elution times of 3.3 min (thiamphenicol), 4.7 min (florfenicol amine) and 5.6 min (florfenicol).

Pharmacokinetic analysis. Standard pharmacokinetic parameters were calculated from the relationship between mean plasma concentrations versus time using the computer program PCNONLIN version 4.2 (Statistical Consultants). The best-fitted models were chosen using Akaike's information criterion estimation (Yamaoka et al. 1978). The area under the concentration time curves (area under curve [AUC]-values) were calculated using the trapezoidal rule. The oral bioavailability was calculated from the AUC-values.

MIC determinations. Three strains (A-21, HI–610, and HI-618) of *Vibrio anguillarum* serotype O2, isolated from cod suffering from vibriosis, were tested for florfenicol MIC. The diseased fish were from the cod culture facilities of either Parisvannet or Austevoll, both research stations of the Institute of Marine Research, Bergen, Norway, and the strains were representative of the strains commonly isolated from cod with vibriosis at these facilities. MIC values were determined using the agar dilution method with some modifications (Washington 1985, Samuelsen & Lunestad 1996). Two bacterial strains, LT25 and LT62, which have known MIC values for florfenicol (8 and 2 µg ml^{-1}, respectively) were used as internal controls according to the protocol by Torkildsen et al. (2000). Strains were maintained on Mueller Hinton Agar (Difco) supplemented with 2% NaCl. Inocula were transferred to Erlen-Meyer bottles with 10 ml Mueller Hinton broth with 2% NaCl and incubated for 48 h in shaking culture at 18°C, giving a final cell density of approximately 5 × 10^8 cells ml^{-1} determined by optical density measurements. Using a 10 µl inoculation loop, bacteria from this broth were distributed on the surface of Mueller Hinton Agar supplemented with 2% NaCl and containing varying concentrations of florfenicol. This testing was performed in triplicate. The agar plates were prepared within 24 h prior to use. The antibacterial agent was added to the agar in 2-fold dilutions from a newly prepared stock solution with an initial concentration of 16 µg ml^{-1} and a final concentration of 0.016 µg ml^{-1}. The temperature of the agar on addition of the agent was 50°C. Following incubation at 18°C for 72 h, the plates were examined for bacterial growth. The lowest concentration of florfenicol at which complete inhibition occurred was recorded as the MIC value.

RESULTS

The standard curves were found to be linear over the range studied (r = 0.99). The limits of quantitation were, based on the standard curves, set to 0.1 µg g^{-1} (ml^{-1}) for florfenicol and 0.2 µg g^{-1} (ml^{-1}) for florfenicol amine in plasma, muscle and liver.

The mean plasma drug concentrations obtained from a single i.v. injection of florfenicol in 6 fish at each sampling point were used to calculate the pharmacokinetic parameters. Data from the i.v.-injected group was best described by a 2-compartment open model with bolus i.v. administration and first-order elimination whereas a 1-compartment model with first-order input and first-order output best described data from the p.o. administration.

Following i.v.-injection, the apparent volume of distribution at steady state, $V_{d(ss)}$ was estimated to be 1.1 l kg^{-1}, the total body clearance, Cl_T, to be 0.015 l kg^{-1} h^{-1}

and the distribution and elimination half-lives, $t_{1/2}\alpha$ and β, to be 1.6 and 43 h, respectively.

Following p.o. administration, the bioavailability (F) was calculated to be 91%, the peak plasma concentration (C_{max}) to be 10.8 µg ml^{-1}, the time to peak plasma concentration (T_{max}) to be 7 h and the $t_{1/2}\beta$ to be 39 h using the computer program. The observed C_{max} and T_{max} in plasma were, however, 12.4 µg ml^{-1} and 9 h with corresponding values of 13.0 µg g^{-1} and 9 h, respectively, in muscle and 12.1 µg g^{-1} and 9 h, respectively, in liver. Calculated $t_{1/2}\beta$ values were 21 and 23 h, respectively, for muscle and liver. The pharmacokinetic parameters in plasma, muscle and liver are listed in Tables 1 & 2, respectively, whereas the mean concentrations versus time curves are shown in Figs. 1 to 4, respectively. The metabolite florfenicol amine was not found in quantitative amounts in plasma or tissues of cod.

The MIC values for florfenicol against the *Vibrio anguillarum* strains tested in this investigation was 0.5 µg ml^{-1} for all strains.

DISCUSSION

Overall, the pharmacokinetic properties of florfenicol in cod are quite similar to the pharmacokinetics of this drug in Atlantic salmon but some important differences exists.

A 1-compartment model with first-order input and first-order output best described the data from the p.o. administration. However, the difference in observed and modelled C_{max} and T_{max} values indicates that the absorption does not strictly follow first-order kinetics. A bioavailability of 91%, a C_{max} value of 10.8 µg ml^{-1} and a T_{max} value of 7 h show that the absorption of florfenicol from the intestine of cod is both fast and close to complete. Similar results are found for this drug in Atlantic salmon by Horsberg et al. (1996), reporting pharmacokinetic parameters of 99% bioavailability, a C_{max} value of 9.1 µg ml^{-1} and a T_{max} value of 6 h. Calculated distribution volumes ($V_{d(ss)}$) of 1.1 l kg^{-1} in cod (this investigation) and 1.32 and 1.12 l kg^{-1} in Atlantic salmon (Martinsen et al. 1993, Horsberg et al. 1996) indicate that the drug is well distributed throughout the body in both species and suggest tissue concentrations similar to those obtained in plasma. This is confirmed for muscle and liver by comparison of the concentration over time curves in Figs. 2, 3 & 4.

Metabolism and elimination of florfenicol differs in cod and Atlantic salmon. The elimination of florfenicol from plasma of cod with a Cl_T of 0.015 l kg^{-1} h^{-1} and $t_{1/2}\beta$ values of 43 h (i.v.-injected group) and 39 h (p.o. administered group) was slow compared with the Cl_T values of 0.086 and 0.047 l kg^{-1} h^{-1} and corresponding $t_{1/2}\beta$ values of 12 and 14.7 h found in Atlantic salmon (Martinsen et al. 1993, Horsberg et al. 1996). Temperature is known to effect the elimination of drugs in fish. However, the small difference in temperature (2°C) between this investigation (8°C) and the investigations of Martinsen et al. (1993) and Horsberg et al. (1996) (10°C) is likely not enough to count for the large difference found. Florfenicol amine is described as a main metabolite of florfenicol in Atlantic salmon and was found in higher concentration in plasma than florfenicol itself from 48 h after administration and throughout the study (Horsberg et al. 1996). In cod, however, florfenicol amine was not detected in quantitative amounts in either plasma or tissues, and the lack of this metabolic pathway may well contribute to the slower elimination of florfenicol in cod than in Atlantic salmon.

With $t_{1/2}\beta$ values of 39, 21 and 20 h, respectively, for plasma, muscle and liver following p.o. administration (Tables 1 & 2) there is a major difference in the elimi-

Table 1. *Gadus morhua*. Calculated pharmacokinetic parameters for florfenicol in cod held in seawater at 8°C following a single intravenous (i.v.) injection and oral (p.o.) administration. $V_{d(ss)}$: volume of distribution at steady state; Cl_T: total body clearance; $t_{1/2}\alpha$: plasma half-life of the drug during distribution phase; $t_{1/2}\beta$: plasma half-life of the drug during elimination phase; MRT: mean residence time; AUC: area under curve; C_{max}: maximum plasma concentration; T_{max}: time of maximum plasma concentration; F: bioavailability

Parameter	i.v. injection	p.o. administration
Dose	10 mg kg^{-1}	10 mg kg^{-1}
$V_{d(ss)}$	1.1 l kg^{-1}	
Cl_T	0.015 l kg^{-1} h^{-1}	
$t_{1/2}\alpha$	1.6 h	
$t_{1/2}\beta$	43 h	39 h
MRT	74 h	
AUC	573 h µg ml^{-1}	524 h µg ml^{-1}
C_{max}		10.8 µg ml^{-1}
T_{max}		7 h
F		91%

Table 2. Calculated pharmacokinetic parameters for florfenicol in muscle and liver of cod held in seawater at 8°C following a single oral administration. $t_{1/2}\beta$: half-life of the drug during the elimination phase; C_{max}: maximum muscle concentration; T_{max}: time of maximum muscle concentration, AUC: area under curve

Parameter	Muscle	Liver
Dose	10 mg kg^{-1}	10 mg kg^{-1}
$t_{1/2}\beta$	21 h	23 h
AUC	459 h µg g^{-1}	405 h µg g^{-1}
C_{max}	13.0 µg g^{-1}	12.1 µg g^{-1}
T_{max}	9 h	9 h

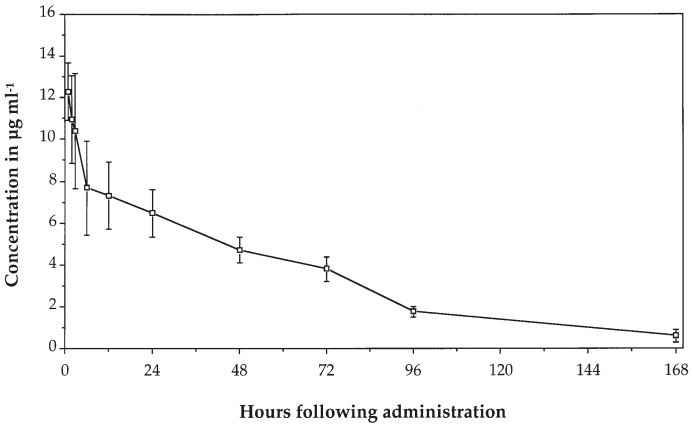

Fig. 1. *Gadus morhua*. Concentrations (mean ± SD; n = 6) of florfenicol in plasma after intravenous injection

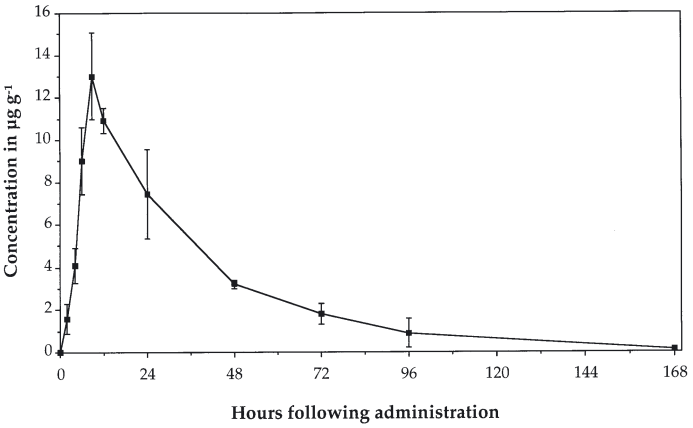

Fig. 2. *Gadus morhua*. Concentrations (mean ± SD; n = 6) of florfenicol in plasma after oral administration

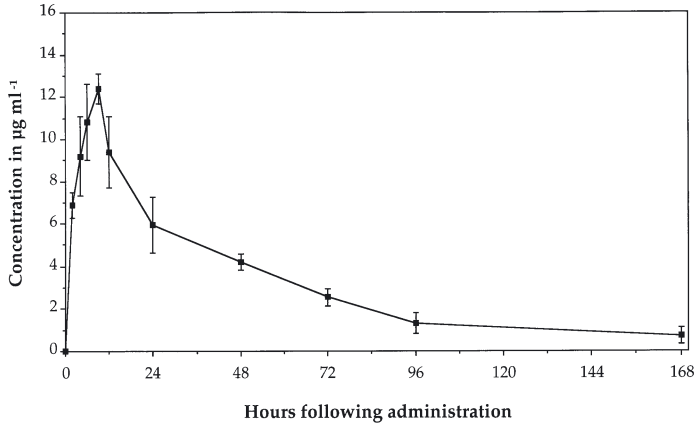

Fig. 3. *Gadus morhua*. Concentrations (mean ± SD; n = 6) of florfenicol in muscle after oral administration

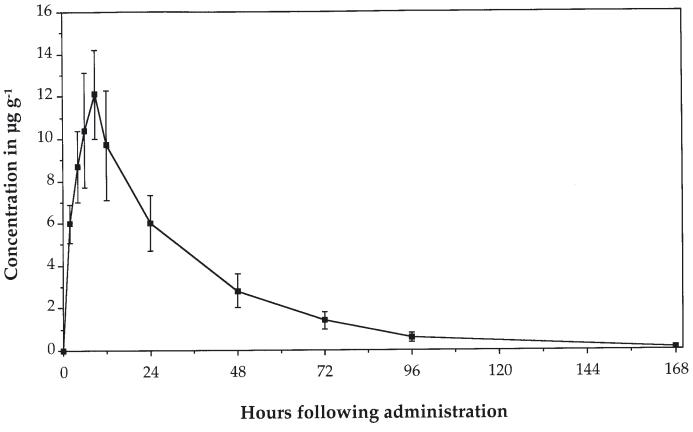

Fig. 4. *Gadus morhua*. Concentrations (mean ± SD; n = 6) of florfenicol in liver after oral administration

nation half-life of florfenicol between plasma and tissues in cod. In comparison, such a difference does not exist for the quinolones, flumequine and oxolinic acid in either cod or Atlantic salmon (Rogstad et al. 1993, Elema et al. 1994, Samuelsen et al. 2000).

An important application of pharmacokinetic data is to establish appropriate treatment regimes. In that respect a much used assumption is that the *in vivo* plasma concentration of the antibacterial agent should exceed the MIC value for the relevant pathogen by a factor of 4, as suggested by Stamm (1989). Recently, Shojaee AliAbadi & Lees (2000) published a paper on dosage regimen optimisation for the treatment of animals with antibacterial agents. They suggested that for a bacteriostatic drug like florfenicol an optimal dosage regimen should maintain concentrations at the site of infection in excess of MIC_{90} for the entire medication period. In this investigation it is shown that florfenicol is rapidly absorbed in cod with a plasma T_{max} value of 7 h and slowly eliminated. Furthermore, in cod given an oral dosage of 10 mg kg^{-1} d^{-1} of florfenicol in feed for 10 consecutive days, the mean (n = 6) plasma and muscle concentrations were 5.0 ± 1.6 µg ml^{-1} and 4.6 ± 0.9 µg g^{-1}, respectively, 24 h following last administration (O. B. Samuelsen unpubl. results). These results indicate that the concentrations in plasma and tissues exceed the MIC value of 0.5 µg ml^{-1} with good margin for the entire medication period (Shojaee AliAbadi & Lees 2000). When applying the recommendation by Stamm (1989), the results from the present investigation show that a single p.o.-administered dose of 10 mg kg^{-1} of florfenicol maintains plasma levels in excess of 2.0 µg ml^{-1}, equal to 4 times an MIC-value of 0.5 µg ml^{-1}, for approximately 3.5 d. Corresponding values for muscle and liver were 3 and 2.5 d, respectively.

The result of the MIC testing from this investigation is within the range reported from a screening of MIC of *Vibrio anguillarum* isolates investigated by Fukui et al.

(1987) and Zhao et al. (1992), who obtained values of 0.4 to 0.8 and 0.2 to 0.8 µg ml^{-1}, respectively. In addition, a study with 10 isolates of *V. salmonicida* reported an MIC to florfenicol of 0.8 µg ml^{-1} (T. Håstein unpubl.). However, investigation of other fish pathogenic bacterial species indicates a broader range of MIC values against florfenicol. From studies with *Aeromonas salmonicida,* (Fukui et al. 1987, Inglis & Richards 1991, Grant & Laidler 1993, Håstein 1994) MIC values in the range of 0.25 to 1.6 µg ml^{-1} were reported. *Photobacterium damselae* (formerly *Pasteurella piscicida)* showed MIC values ranging from 0.004 to 0.6 µg ml^{-1} (Fukui et al. 1987, Kim & Aoki 1993), whereas the MICs for *Edwardsiella tarda* isolates ranged from 0.4 to 1.6 µg ml^{-1} (Fukui et al. 1987). Displaying large variability with respect to this parameter, the MIC of florfenicol against 48 *Flavobacterium psychrophilium* isolates ranged from 0.00098 to 16 µg ml^{-1} (Rangdale et al. 1997). Furthermore, a wide range of bacterial strains, mainly *Vibrio* spp., *Aeromonas* spp. and *Pseudomonas* spp. isolated from scallop *Pecten maximus* larvae, had MIC values ranging from 0.13 to 8 µg ml^{-1} when a medium with 2% NaCl was applied (Torkildsen et al. 2000). When a medium based on seawater diluted to 25 ppt salinity was used, they found that the MIC for the same strains ranged from 0.25 to >16 µg ml^{-1}, indicating reduced antibacterial activity when florfenicol was dissolved in seawater.

In summary, the pharmacokinetic data obtained in this investigation indicate florfenicol to be a qualified candidate for treatment of bacterial infections in cod. The high concentration of the drug in both plasma and tissues and slow elimination are excellent properties from a therapeutic point of view. However, due to the reported low effectivity in a field situation, efficacy studies under controlled conditions and additional field studies should be performed before a recommendation can be made.

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