

Disposition of ^{14}C -flumequine in eel *Anguilla anguilla*, turbot *Scophthalmus maximus* and halibut *Hippoglossus hippoglossus* after oral and intravenous administration

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ABSTRACT: The absorption, distribution and elimination of ^{14}C -labelled flumequine were studied using whole body autoradiography and liquid scintillation counting. Flumequine was administered to eel *Anguilla anguilla*, turbot *Scophthalmus maximus* and halibut *Hippoglossus hippoglossus* intravenously and orally as a single dose of 5 mg kg^{-1} , corresponding to 0.1 mCi kg^{-1} . The turbot and halibut studies were performed in salt water (salinity of 32‰) at temperatures of $16 \pm 1^\circ\text{C}$ (turbot) and $9.5 \pm 0.5^\circ\text{C}$ (halibut). The eel study was conducted in fresh water at $23 \pm 1^\circ\text{C}$. In the intravenously administered groups flumequine was rapidly distributed to all major tissues and organs. After oral administration flumequine also appeared to have rapid and extensive absorption and distribution in all 3 species. After the distribution phase, the level of flumequine was higher in most organs and tissues than in the blood, except in muscle and brain. The most noticeable difference between the species was the slow elimination of flumequine from eel compared to turbot and halibut. In orally administered eels, substantial amounts of flumequine remained in all major organs/tissues for 7 d. At 28 d significant levels of flumequine were present in liver, kidney and skin (with traces in muscle), and at the last sampling point (56 d) in eye, bone, bile and posterior intestine. In orally administered turbot significant levels of flumequine were observed over 96 h in bile, urine, bone, skin, intestine and eye, and traces were detected over 28 d in bone and eye in addition to a significant level in bile. In orally administered halibut, significant levels of flumequine were observed in bile, skin, intestine and eye over 96 h. Traces were present in skin and eye over 7 d. The maximal flumequine concentrations in blood were calculated to be $2.5 \text{ mg equivalents l}^{-1}$ (eel at 12 h), 0.8 mg l^{-1} (turbot at 6 h) and 0.6 mg l^{-1} (halibut at 6 h) after oral administration.

KEY WORDS: Flumequine · Pharmacokinetics · Disposition · Tissue distribution · Eel · Turbot · Halibut · Whole body autoradiography

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INTRODUCTION

Eel *Anguilla anguilla* and turbot *Scophthalmus maximus* are 2 important species in European aquaculture production. Eel is a catadrome fish species cultivated in temperate (20 to 26°C) fresh water, whereas turbot is

a marine species cultivated in temperate (15 to 18°C) seawater. The European production volumes of eel and turbot are approximately 11 000 and 5000 tons per year, respectively. Halibut *Hippoglossus hippoglossus* is a marine cold-water species (8 to 10°C) currently being developed for aquaculture in Iceland, Norway, Scotland and Canada. The annual production of halibut in these countries is currently approximately

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750 000 fry, that may produce approximately 2500 t when slaughtered.

The farming of these 3 species has already encountered problems with several bacterial infections, such as vibrio infections (*Vibrio anguillarum* and *Vibrio vulnificus*) and atypical *Aeromonas salmonicida* infections (Møllergaard & Dalsgaard 1987, Noga et al. 1990, Biosca et al. 1991, Fouz et al. 1992, 1995, Austin et al. 1993, Liewes & Haenen 1993, Pedersen et al. 1994, 1999, Grisez et al. 1996, Larsen & Pedersen 1996, Bergh et al. 1997, Olsson et al. 1998, Bricknell et al. 1999, 2000, Dalsgaard et al. 1999, Gatesoupe et al. 1999).

One commonly used antimicrobial agent in aquaculture is flumequine. Flumequine is a broad-spectrum synthetic antimicrobial agent belonging to the 4-quinolones and has the properties of a weak acid. Plasma disposition of flumequine after intravenous and oral administration have been studied in Atlantic salmon *Salmo salar* (Rogstad et al. 1993, Elema et al. 1995, Martinsen & Horsberg 1995), halibut (Samuelsen & Ervik 1997, Hansen & Horsberg 1999), turbot (Hansen & Horsberg 1999), eel (Hansen & Horsberg 2000a), cod *Gadus morhua* and wrasse *Ctenolabrus rupestris* (Hansen & Horsberg 2000b) and channel catfish *Ictalurus punctatus* (Plakas et al. 2000). Plakas et al. (2000) also studied the tissue distribution and the metabolism of flumequine.

The aim of this study was to perform a comparative investigation on flumequine disposition in eel, turbot and halibut. Whole body autoradiography and liquid scintillation counting (LSC) was used to study the disposition and tissue distribution. Studies on flumequine using whole body autoradiography techniques have not been published previously. This technique is very valuable for visualizing the disposition and retention of drugs.

MATERIALS AND METHODS

Test substance. ^{14}C -labeled flumequine was produced in 1995 by Dupont NEN Products, Boston, MA, USA, with a radiochemical purity of 99%. When tested before the study, the purity was only 92%. It was therefore purified by means of solid phase extraction on a column of the Bond Elut type with C2 sorbent material to a radiochemical purity of >98%.

Test facilities and test fish. The eel *Anguilla anguilla* study was conducted at The National Veterinary Institute, Oslo, Norway. The eel had been caught in the Arendal area, weighed 104 ± 12 g (mean \pm SD) and were held in fiberglass tanks (240 l) supplied with running fresh water at a temperature of $23 \pm 1^\circ\text{C}$ (pH 6.5; Ca: 2.75 mg l^{-1} ; Mg: 0.48 mg l^{-1}). The turbot *Scoph-*

thalmus maximus and halibut *Hippoglossus hippoglossus* studies were conducted at the NIVA Marine Research Station, Solbergstrand, Drøbak, Norway. The fish were kept in fiberglass tanks (600 l) supplied with running seawater (pH 8, salinity 32‰) at temperatures of $16 \pm 1^\circ\text{C}$ (turbot) and $9.5 \pm 0.5^\circ\text{C}$ (halibut). The turbot and halibut were approximately 1 yr old (farmed) and weighed 78 ± 13 g (turbot) and 69 ± 18 g (halibut). The experiments were approved by the institutional animal use committee.

Intravenous and oral administration. ^{14}C -labelled flumequine was administered orally and intravenously at a dose of 86 and 82 $\mu\text{Ci kg}^{-1}$, respectively, corresponding to 5 mg flumequine kg^{-1} in each group after addition of cold flumequine. The flumequine for intravenous administration was dissolved in saline, pH 10. The injection volume was 2 ml kg^{-1} . For oral administration flumequine was suspended in corn oil.

During the adaptation period the fish were fed a commercial pelleted fish diet. Feed was withheld 1 d prior to drug administration and feeding was resumed the following day.

In the group given the flumequine intravenously, each fish was netted from the acclimatization tank and individually weighed in a small tank of water. The fish were then anaesthetized with benzocaine (50 mg l^{-1} water). The intravenous injection of flumequine was accomplished by placing the fish on damp paper and the flumequine solution was slowly injected into the caudal vein using a 1 ml disposable syringe and a 0.5×16 mm needle (Terumo, Leuven, Belgium). The position of the needle was confirmed by aspiration of blood before, during and after the injection. Fish in which the needle dislocated during the injection were discarded and replaced. Each fish was dosed according to individual weights.

In the group given the flumequine orally, each fish was netted from the acclimatization tank and individually weighed. The drug was administered through polyethylene stomach tubes. In turbot and halibut, each fish was manually restrained without anaesthesia at the drug administration. The eel was lightly anaesthetized with benzocaine (50 mg l^{-1} water) before administration. The stomach tubes were each filled with 5 pellets of fish feed (2.5 mm). Before administration, the test suspension was mixed for 15 s on a whirl mixer and the dose was transferred to the stomach tube using a pipette. The pellets in the tube were coated with the test suspension and were used to detect regurgitation and facilitate the deposit of the drug suspension in the stomach (Horsberg 1994). Each fish was dosed according to individual weights. After drug administration, all orally dosed fish were transferred to individual tanks for observation of regurgitation before being transferred to the experimen-

tal tanks. No regurgitation was observed (as indicated by oil droplets on the water surface or pellets in the water) during the 5 min in the observation tanks.

A discard permit for the study was obtained from the Institute of Radiation Hygiene in Norway. To minimize the effluents of ^{14}C , the outlet water from the fish tanks were filtrated and all solid effluents removed.

Sampling. In the intravenously administered groups samples were collected at 12 h, 48 h, 96 h, 14 d, 28 d and 56 d post administration. In the orally administered groups, samples were collected at 6 h, 12 h, 24 h, 48 h, 96 h, 7 d, 14 d, 28 d and 56 d post administration. At each sampling time 2 fish were sacrificed by an overdose of benzocaine (200 mg l^{-1} for 10 min) and frozen before being embedded in individual blocks of cooled 1% solution of sodium carboxymethyl cellulose in water (0°C) followed by immediate freezing with dry ice in n-hexane (-75°C).

Analytical procedures. Each sample was analysed using a standard technique for whole body autoradiography and related studies on radioactive isotopes (Ullberg 1954, 1977). Sagittal sections (20 to 40 μm) from the whole fish were obtained on tape (No. 821, 3M Co., St. Paul, MN, USA) at -20°C in a PMV cryomicrotome (PMV; 450 MP, Stockholm, Sweden). The sections were freeze-dried overnight, before application on Structurix D7 (Agfa, Antwerp, Belgium) for autoradiography. The films were exposed at -20°C for approximately 3 mo before developing.

From the material remaining in the frozen blocks, samples weighing 10 to 100 mg were obtained from muscle, brain, blood, kidney, liver, bile and skin. The samples were digested with 2 ml soluene (Packard, Holland) at 37°C . Then 10 ml of liquid scintillation cocktail (Hionic Fluor, Packard) was added to each vial. The radioactivity in the samples was counted in a Packard Tri-Carb 1900CA LSC analyzer, using a Packard automatic quenching standard to control the counting efficacy.

Pharmacokinetic modelling. Pharmacokinetic modelling was performed in the intravenous-group using the computer program WIN-NONLIN, version 1.1 (Statistical Consultants Inc., Lexington, KY), in a least square non-linear regression analysis. Standard pharmacokinetic parameters were calculated according to a non-compartment model. The intercept with the y-axis was calculated by back-extrapolation of the curve, using the first 2 data points. The terminal elimination-rate constant, λ_z , was estimated according to the algorithm of Dunne (1985). The concentration versus time curve

was extrapolated to infinity using λ_z . In halibut and turbot the 2 last sample points were omitted due to rising flumequine concentrations.

RESULTS

Liquid scintillation counting

Oral administration

The concentrations of flumequine equivalents (radioactivity) versus time in different organs are presented in Fig. 1 (eel *Anguilla anguilla*), Fig. 2 (turbot *Scophthalmus maximus*) and Fig. 3 (halibut *Hippoglossus hippoglossus*). The highest concentrations of radioactivity were found in bile of eel and turbot (in halibut no samples of bile were available). Eel showed higher levels and slower elimination of radioactivity in all tissues compared to turbot and halibut. In turbot and halibut the radioactivity declined relatively rapidly in all tissues over 7 d. After 7 d there was a small increase in radioactivity level in most tissues in halibut and turbot.

The relative distribution of radioactivity between blood and tissues at 6 and 48 h after oral administration is presented in Table 1. In all 3 species, the level of radioactivity was generally lower in muscle and brain than in blood, and higher than blood in kidney, liver and skin. The highest level compared to blood was found in bile.

Intravenous administration

The pattern of radioactivity in the intravenously administered fish was generally similar to the orally administered fish, but with higher levels. In non-

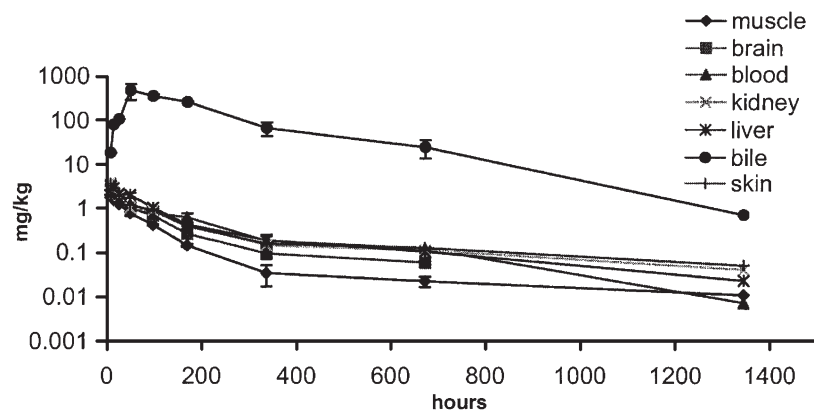


Fig. 1. Mean (\pm SEM) plasma concentration profiles of flumequine equivalents in different tissues of eel *Anguilla anguilla* ($n = 2$) after a single 5 mg kg^{-1} dose administered orally

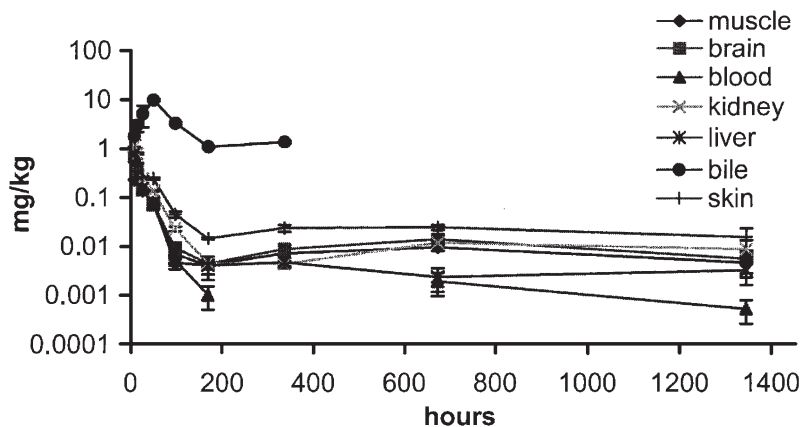


Fig. 2. Mean (\pm SEM) plasma concentration profiles of flumequine equivalents in different tissues of turbot *Scophthalmus maximus* ($n = 2$) after a single 5 mg kg^{-1} dose administered orally

compartment modelling of the LSC data from eel, a distribution volume at steady state (V_{ss}) of 1.2 l kg^{-1} , a terminal elimination half-life ($T_{1/2} \lambda_z$) of 232 h and a clearance (Cl) of $0.006 \text{ l h}^{-1} \text{ kg}^{-1}$ were calculated. In turbot V_{ss} of 0.8 l kg^{-1} , $T_{1/2} \lambda_z$ of 38 h and a Cl of $0.043 \text{ l h}^{-1} \text{ kg}^{-1}$ were calculated and in halibut V_{ss} of 3.8 l kg^{-1} , $T_{1/2} \lambda_z$ of 62 h and a Cl of $0.058 \text{ l h}^{-1} \text{ kg}^{-1}$ were calculated.

Whole-body autoradiography

Oral administration

The results were in good agreement with those from the LSC study. In addition to the tissues sampled for LSC the whole body autoradiographic (WBA) study revealed radioactivity in the intestine, eye, gills, bone, heart, spleen and urine. In eel the radiolabelling in these tissues remained high from 6 h through 96 h (Fig. 4.). During the rest of the experimental period the levels declined gradually and radioactivity was still recorded at 14 d in the gills and at 56 d in the bone, eye and intestine.

In turbot high levels of radiolabelled compound were present in the intestine, eye, gills, bone, heart, spleen and urine at 6 h. The radioactivity in bone increased from 6 h through 24 h. For the other organs mentioned the concentration of radioactivity decreased slightly during this period. At 48 h radioactivity still remained at a high level in bone, eye, intestine and urine (Fig. 5). During the rest of the study period the levels decreased gradually. Radioactivity in

bone, however, was still detectable at 56 d.

In halibut the levels of radioactivity were largely similar to that of turbot at 6 h except for the gills and bone, which contained a considerably lower concentrations of radiolabelled compound than in turbot. The levels of radioactivity declined gradually during the rest of the study. Radioactivity was still detectable in the eye and intestine at 96 h (Fig. 6).

Intravenous administration

The results largely confirmed the observations from the oral study. Accordingly, the general retention time was substantially longer in the eel compared to turbot and halibut. Furthermore, the concentrations were higher and the retention times longer in both bone and gills of turbot compared to halibut.

DISCUSSION

At each time point only 2 fish were sampled. Pharmacokinetic modelling using the liquid scintillation count (LSC) data could, due to the low number of samples at each time point and normal inter-individual variation, give biased results. For this reason we will only briefly discuss calculated pharmacokinetic parameters from these data. Despite this methodological limitation, important information on the disposition of flumequine in the different fish species was obtained. In the following discussion, the results refer to the orally administrated fish unless otherwise specified.

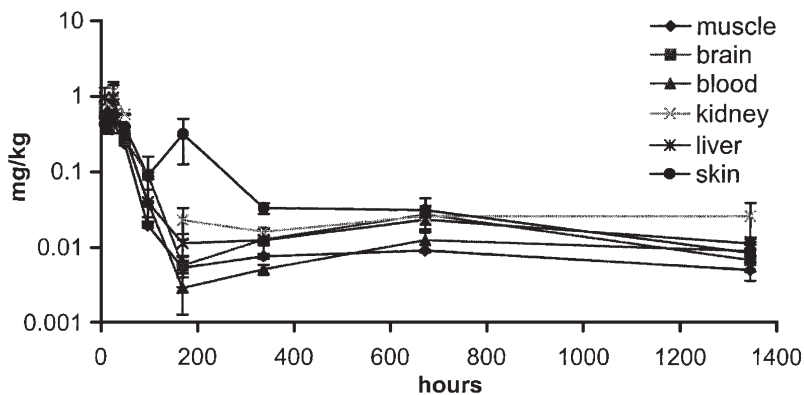


Fig. 3. Mean (\pm SEM) plasma concentration profiles of flumequine equivalents in different tissues of halibut *Hippoglossus hippoglossus* ($n = 2$) after a single 5 mg kg^{-1} dose administered orally

Table 1. Tissue:blood ratio of total ^{14}C -flumequine residues after a 5 mg kg^{-1} orally administered dose (average of 2 samples). -: No sample available

Species	Tissue	Tissue:blood ratio	
		(6 h)	(24 h)
Eel	Muscle	0.79	0.63
	Brain	-	0.83
	Liver	1.37	1.64
	Kidney	1.55	0.84
	Skin	1.52	0.99
	Bile	7.75	390
Turbot	Muscle	0.30	1.07 ^a
	Brain	1.03	1.03 ^a
	Liver	1.04	1.09
	Kidney	1.50	1.91
	Skin	1.12	3.41
	Bile	2.34	134
Halibut	Muscle	0.76	0.75
	Brain	0.99	0.86
	Liver	1.40	0.96
	Kidney	1.27	1.83
	Skin	0.62	1.25

^aOf the 2 samples, one was higher than blood, the other lower

The elimination half-lives estimated from the LSC of blood (intravenously administered fish) are in the same range as reported from other flumequine studies in the same species (Boon et al. 1991, van der Heijden et al. 1994, Samuelsen & Ervik 1997, Hansen & Horsberg 1999, 2000a,b). Turbot seem to have elimination half-lives in the same range as Atlantic salmon *Salmo salar*, which have been reported to have elimination half-lives of 29 to 40 h (Rogstad et al. 1993, Elema et al. 1995). The elimination half-life of halibut (62 h) seemed to be a little slower compared to Atlantic salmon.

The high and persistent flumequine level in bile, intestine and urine, and at earlier sampling times in kidney, liver and gills reflects that these are the main excretory organs/routes. The gills are regarded as one of the most important sites of elimination of xenobiotics in fish, due to the large surface area and blood flow together with the short distance between the blood and water. Accordingly, Sohlberg et al. (1999) reported that Atlantic salmon *Salmo salar* excrete approximately 60% of an administered dose of flumequine through the gills, in addition to excretion to urine, bile and faeces.

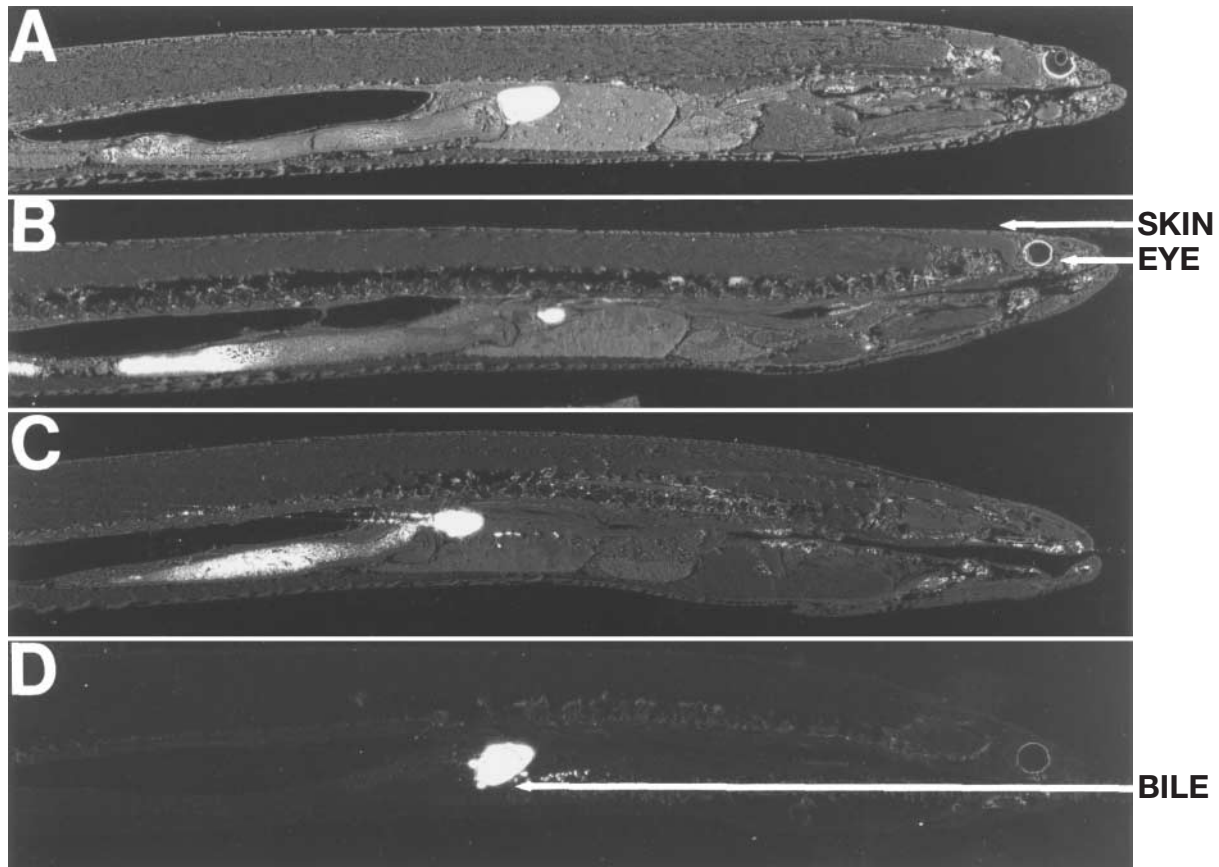


Fig. 4. Whole body autoradiograms showing the tissue distribution pattern of flumequine in eel *Anguilla anguilla* after a single oral administration of ^{14}C -flumequine. The samples were taken after (A) 24 h, (B) 48 h, (C) 96 h and (D) 672 h. White areas correspond to high levels of radiolabelled compound

Analysing various tissues for flumequine and comparing tissue: blood (t:b) ratios we found that muscle had the lowest t:b ratio in all 3 species (Table 1). In eel *Anguilla anguilla*, van der Heijden et al. (1993) found a higher muscle:plasma ratio (0.99 at 48 h) than our muscle: blood ratio, a lower liver:plasma ratio, and significantly lower kidney:plasma and skin:plasma ratios at 48 h, than observed in our study. The eel used in the study of van der Heijden et al. (1993) were approximately 470 versus 100 g in our study, and the flumequine was administered intramuscularly. Difference in size, nutritional status and the different way of administration may have contributed to these discrepancies. Eel muscle may contain more than 30% fat, and van der Heijden et al. (1993) found high levels of flumequine in fat.

The skin: blood ratios were 1.0 (eel), 3.4 (turbot *Scophthalmus maximus*) and 1.3 (halibut *Hippoglossus hippoglossus*) at 48 h. Malvisi et al. (1997) reported ratios of 5.22 and 4.6 for skin: muscle and vertebra: muscle respectively at 24 h in sea bream *Sparus aurata*. In the present study, bone was not sampled for LSC, but high affinity of flumequine to bone was seen on the autoradiograms. The high affinity for bone surface, especially seen in turbot (Fig. 5), can be explained by the ability of flumequine to complex with divalent ions (like Ca^{2+} and Mg^{2+}). The affinity of flumequine for skin can be explained by melanin's capacity to bind drugs (Potts 1963, Ullberg et al. 1970, Lindquist & Ullberg 1972, Larsson 1993, Fukuda & Sasaki 1994, Howells et al. 1994, Salazarbookaman et al. 1994). Several studies have reported residues of flumequine and other fluoroquinolones in bone and skin for a long time after the level is below detection in plasma/muscle (Steffenak et al. 1991, van der Heijden et al. 1993, Elema et al. 1994, Martinsen et al. 1994).

The high flumequine affinity for the eye mainly involved the chorio-retina, but also the surface of the lens

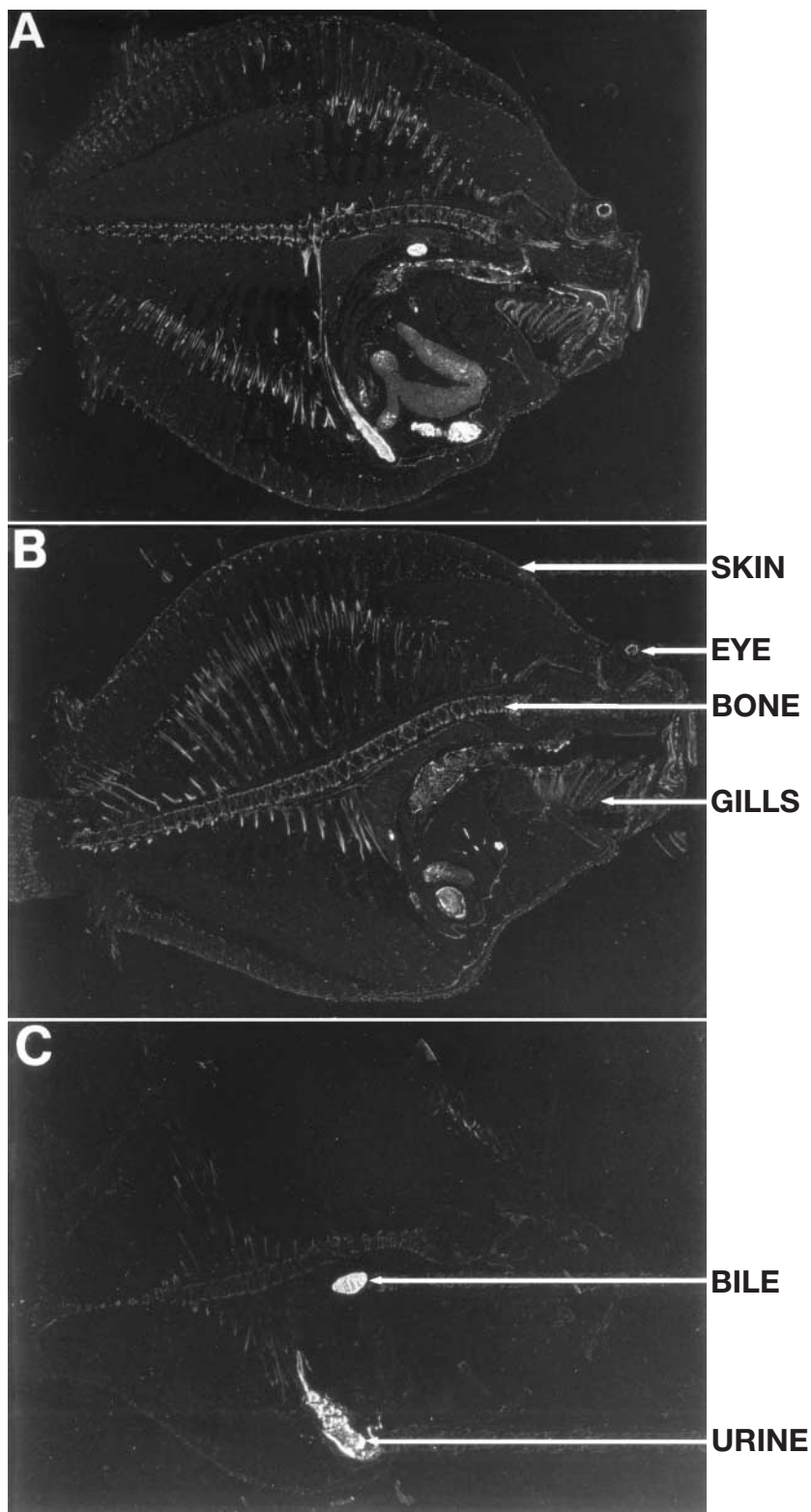


Fig. 5. Whole body autoradiograms showing the distribution pattern of flumequine in turbot *Scophthalmus maximus* after a single oral administration of ^{14}C -flumequine. The samples were taken after (A) 24 h, (B) 48 h and (C) 96 h. White areas correspond to high levels of radiolabelled compound

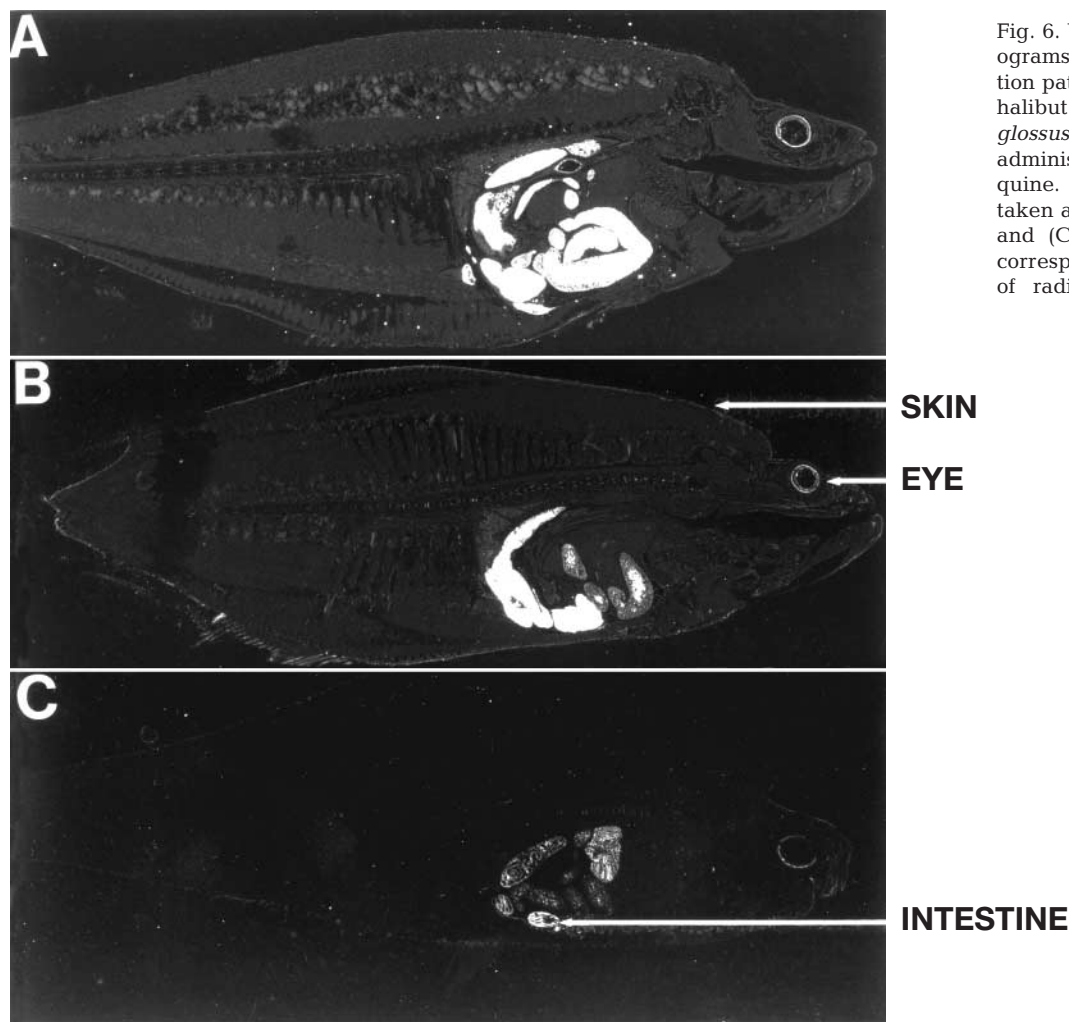


Fig. 6. Whole body autoradiograms showing the distribution pattern of flumequine in halibut *Hippoglossus hippoglossus* after a single oral administration of ^{14}C -flumequine. The samples were taken after (A) 24 h, (B) 48 h and (C) 96 h. White areas correspond to high levels of radiolabelled compound

showed a high level of radioactivity (Fig. 4A). Binding to melanin in rabbit eyes has been shown for the fluoroquinolone sparfloxacin by Fukuda & Sasaki (1994). Binding of flumequine to lens surface has not been described previously and the mechanism for this binding is not known.

A relatively extensive distribution to the central nervous system (CNS) was observed in all 3 species, and may explain several cases where CNS-symptoms including mortality have been observed in fish after administration of flumequine (Scallan 1983, O'Grady et al. 1988, Hiney et al. 1994, Hansen & Horsberg 2000b). CNS symptoms have also been observed in other animal species and have been related to the passage of the drug through the blood-brain barrier (Mevius et al. 1990).

In all 3 species studied we found maximal tissue levels (T_{\max}) at 6 h, (the first sampling point) except for bile. In eel and turbot we found a T_{\max} in bile of 96 h (455 mg l^{-1}) and 48 h (10 mg l^{-1}), respectively. Van der Heijden et al. (1993) reported T_{\max} of flumequine

(HPLC) from different tissues in eel of 48 h to 8 d after intra muscular administration. Plakas et al. (2000) reported a T_{\max} in bile at 72 h (119 mg l^{-1}) in channel catfish *Ictalurus punctatus*.

In eel significant levels of flumequine were still seen at the last sampling time (56 d) in bone, bile, eye and bone on WBA. Different pharmacokinetic properties of quinolones in freshwater compared to seawater (Ishida 1992, Elston et al. 1994) probably contributes significantly to the observed difference in flumequine elimination. Comparing eel to turbot and halibut, the ion-trapping of flumequine in the gut by Mg^{2+} and Ca^{2+} ions from seawater makes the environment for both primary absorption and entero-hepatic cycling more favorable in fresh water. The high concentration of flumequine excreted in bile is more likely to be reabsorbed from fish in freshwater compared to saltwater, and contributes to the explanation for the slow elimination of flumequine from eel compared to turbot and halibut. The slow elimination rate in eel, also compared to other fresh water species (van der Heijden et

al. 1994), has been related to the small gill surface area of eel (Boon et al. 1991), the gills being the major excretory organ for xenobiotics in fish.

The relatively large distribution volume in all 3 species, the high $T_{1/2}$, λ_z and low clearance in eel compared to turbot and halibut as calculated by non-compartment modeling, is in accordance with previous studies on flumequine pharmacokinetics (van der Heijden 1994, Samuelsen & Ervik 1997, Hansen & Horsberg 1999, 2000a,b). In eel the level of radioactivity decreased in tissues and organs during the whole study period. In turbot and halibut the level increased somewhat in most tissues and organs from the 14 d sampling point. It is important to remember that the tissue levels in turbot and halibut at these sampling times were low (Figs 5 & 6), 3 to 100 orders of magnitude lower than observed in eel. Previous studies of flumequine pharmacokinetics have also shown an increase of flumequine levels in the terminal depletion phase (Hansen & Horsberg 1999). Steffenak et al. (1991) reported an increase in liver oxolinic acid concentration in the late depletion phase after salmon was subjected to stress, and also demonstrated leakage from flumequine reservoirs when the salmon was subjected to cooking (Steffenak et al. 1994). Release of flumequine from bone and other reservoirs may therefore explain the small rise in the flumequine level in the terminal depletion phase found in our study.

In our study we did not determine metabolites qualitatively or quantitatively. The radioactivity observed represents the total flumequine residue level consisting of both parent compound and metabolites. Several studies have shown that metabolism of flumequine in various fish species appears to be slow with low levels of metabolites being detected (van der Heijden et al. 1993, 1994, Samuelsen & Ervik 1997, Plakas et al. 2000). With the exception of bile and urine, the total flumequine residues shown in WBA and measured by LSC in this study, therefore most likely to a large extent represent the parent compound.

Acknowledgements. The authors express their appreciation for the skilful technical assistance of Mrs Inger Lise Gross. This study was supported by the Norwegian Research Council, grant no. 111333/100.

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Editorial responsibility: Otto Kinne (Editor), Oldendorf, Germany

Submitted: February 28, 2001; Accepted: July 31, 2001
Proofs received from author(s): November 28, 2001