Bull. Environ. Contam. Toxicol. (2005) 75:109–114

DOI: 10.1007/s00128-005-0725-9

Bulletin of Environmental Contamination and Toxicology

Evidence of Veterinary Drug Residues in Slovenian Freshwater Fish

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Received: 21 October 2004/Accepted: 4 April 2005

Fish farming is usually a monoculture, and the living conditions of farmed fish are essentially different from those in nature. In fish farming, a high density of individual fish is present in ponds, basins and particularly tanks. As a result of this high fish density, the transmission of infectious diseases caused by bacteria, parasites and fungi is much higher than in fish living under natural circumstances. As in many other intensive farming systems, the use of antimicrobial compounds in aquaculture to prevent or treat fish diseases is necessary. Treatment of cultured fish is characterized by mass-medication and, in many cases, through water-borne exposure. One of the major differences from warm-blooded husbandry animals, such as cattle, pigs or poultry, is the apparent accumulation and persistence of veterinary drug residues in cold-blooded fish species as a consequence of a slower metabolism. Furthermore, the therapeutic doses administered in aquatic animals are usually much higher than in warm-blooded animals because of lower bioavailability. For the setting of withdrawal times to reach acceptable drug residue levels in fish, it must be recognized that there is large variation in the pharmacokinetic parameters between fish species, accumulation of drugs, different tissues and the external influence of parameters such as water temperature. As can be see from data in the literature, some drugs accumulate in the liver. Baradat and co-workers (1993) found the highest concentration of chloramphenicol in the livers of rainbow trout. Virginiamycin also accumulates in the liver, followed by the skin and kidneys, while the concentration is low in muscle (Cravedi 1990). Other drugs accumulate in the skin; for example, sulphadiazine. At zero withdrawal time, the highest concentration is found in the skin, followed by the liver and kidneys, while the lowest amount is detected in muscle (Intorre et al. 1996). The behaviour of tetracyclines in fish is somewhat different. They are competitively deposited with calcium in newly forming bones, but high levels have also been found in the liver, followed by the skin, kidneys and muscle (Weber and Ridgeway 1962; Nouws et al. 1993). Withdrawal periods for fish can vary from 90 days (as for oxytetracycline at water temperatures below 6° C, Jacobsen 1989) to five days in the case of some antibiotics (Cravedi 1990; Zimmermann and Büning-Pfaue 1993). On account of long withdrawal periods for some drugs, there is a real possibility for the occurrence of their residues in fish. Murray and co-workers (1987) monitored the residues of oxytetracycline in trout

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muscle and, in 7 of 54 samples of fish taken from the market, found oxytetracycline ranging from 8 to 37 ng/g. In all instances, the concentrations of oxytetracycline were considerably lower than the permitted maximum residue level (MRL) for tetracyclines – that is, 100 ng/g.

As elsewhere in the world, intensive freshwater fish farming has been increasing in Slovenia. However, one must take into account that water quality is important for fish farming and could be also a burden to the environment. For their optimum life requirements, each fish species prefers different water qualities. Dobeic and co-workers (2000) surveyed the quality of water in salmonid fish farms in Slovenia with the aim of evaluating it as a cause of fish health problems. Most of the tested values were within the limits of the permitted standards for fish breeding, with the exception of ammonia, which was higher than that allowed in most cases.

The present study was designed to assess the presence and safety levels of some drugs approved for use in fish farms. In addition, this study also examined fish samples for chloramphenicol, which is banned from animal feed.

MATERIALS AND METHODS

From 1995 to 1999, freshwater fish samples were taken from 77 fish farms. We analyzed 194 different samples (4–8 fish/sample), from which 157 (81%) were various species of trout such as brown trout (*Salmo trutta fario)*, rainbow trout (*Salmo gairdneri)* and marble trout (*Salmo marmoratus)*, 24 samples of common carp *(Cyprinus carpio)*, 4 pike (*Esox lucius*), 3 pikeperch (*Stizostedion lucioperca)*, 2 grass carp (*Ctenopharyngodon idella)*, 1 wels (*Silurus glanis),* 1 perch (*Perca fluviatilis*) and 2 bighead carp (*Hypophthalmichthys sp.*) (Table 1).

Table 1. Types and number of fish analyzed for veterinary drug residues

Year	Trout *Salmo*	Carp *Carpio*	Pike *Esox lucius*	Pikeperch *Stizostedion lucioperca*	Wels *Silurus glanis*	Perch *Perca fluviatilis*	Other
1995	24						
1996	16	4					
1997	27	2					2[a]
1998	28	6					1[b]
1999	62	12	4	3	1	1	1[b]

a grass carp, *Ctenopharyngodon idella*
b bighead carp, *Hypophthalmichthys sp.*

The selected fish tissue was muscle and skin in natural proportion as demanded in Council Regulation (EEC) No 2377/90 (Off J Eur Commun 1990). All samples

were prepared immediately after receipt. The fish were gutted and filleted. For antibiotic analyses, a small piece was taken from each fish fillet and combined by compressing in a juicer to obtain a fluid. The analyses were performed immediately or on the following day (fluid storage at 4° C). For other analyses, the rest of the fillets were minced with a Büchi-400 homogenizer, pooled and thoroughly mixed. Subsamples were kept under -18° C until the day of analysis.

The analyses were performed in series of 6 to 12 samples. All demands for quality control regarding the residue analysis of drugs were followed, which include one negative and one spiked sample per series for controlling the performance of the analyses. All positive samples were reanalyzed in duplicates.

Antibiotics in fish samples were detected and confirmed by microbiological assays. For microbiological assays (Anonymous 1974), we used Antibiotic Seed Agar A1 from Biolife® as a medium with the addition of the appropriate test culture sensitive to the assessed antibiotic. The test cultures were *B. cereus* ATTC 11778 and *St. epidermidis* ATCC 12228 for detection of tetracyclines, *S. luteae* ATCC 9341 for determination of penicillins and tylosin, *S. luteae* ATCC 9341 and *S. luteae* ATCC 15957 for determination of erythromycin, *B. subtilis* ATTC 6633 and *S. luteae* ATCC 9341 for determination of streptomycin, and *St. epidermidis* ATCC 12228 and *S. luteae* ATCC 9341 for determination of neomycin. The samples were warmed at 80° C for 5 minutes and inoculated into metal cylinders placed on the prepared seeded agar. The inoculum penetrated into the agar and, if the antibiotics were present in the sample, the growth of the test bacteria was inhibited. The detection limits were different for each antibiotic and were as follows: tetracycline and oxytetracycline 0.05 μg/mL, chlortetracycline 0.01 μg/mL, penicillin 0.013 I.E., tylosin 0.1 μg/mL, erythromycin 0.05 μg/mL, streptomycin 0.1 μg/mL and neomycin 0.25 μg/mL.

Chloramphenicol was analyzed by GC-ECD. After the addition of an internal standard (*meta* isomer of chloramphenicol) to fish samples, chloramphenicol was extracted with water using Ultra-turrax and ultrasonic energy. Fat was removed with hexane and the procedure was followed by a cleanup on Extrelut® columns. Chloramphenicol was eluted in ethyl acetate, evaporated to dryness and re-dissolved in water. Fat was separated with hexane again. Chloramphenicol residues were derivatized by silylation to form a thermally stable product, which was analyzed by capillary GC with EC detector. Limit of detection was 1 ng/g (Cerkvenik 2002).

Sulphonamides were determined by HPTLC. Before extraction of sulphonamides from fish samples with ethyl acetate, the internal standard (sulphapyridine) was added. Clean-up was performed by solid-phase extraction (SPE) using silica columns. Sulphonamides were eluted with a mixture of methanol and acetonitrile. After evaporation to dryness under nitrogen, the residues were re-dissolved in methanol. Sulphonamides were separated from the co-extracts using HPTLC. Pre-

adsorbent layer silica gel HPTLC plates were used. Developed plates were treated with fluorescamine and the intensities of the visible spots were evaluated using a fluorescence densitometer at 410 nm (Van Poucke et al. 1991). With the analytical procedure described, we were able to monitor: sulphamerazine, sulphadiazine, sulphadimethoxine, sulphamethazine, sulphamonomethoxine, sulphathiazole, sulphamethoxazole, sulphaquinoxaline, sulphapyridine, sulphachloropyridazine, sulphadoxine, sulphanilamide, sulphamethoxypyridazine, sulphamoxole, sulphisoxazole and sulphaphenazole. Quantification of positive samples was performed using a matrix-matched calibration curve. The limits of detection for individual sulphonamides ranged from 10 to 20 ng/g.

RESULTS AND DISCUSSION

In 194 samples of farmed freshwater fish, we performed 174 analyses for antibiotics, 97 analyses for chloramphenicol and 176 analyses for sulphonamides (Table 2).

Table 2. Number and type of analyses performed in farmed freshwater fish in the years 1995 to 1999.

Type of analyses	No. of tests	Negative samples	Positive samples
Antibiotics	174	174	0
Chloramphenicol	97	97	0
Sulphonamides	176	171	5

No positive samples of antibiotics (tetracyclines, penicillins, tylosin, erythromycin, streptomycin or neomycin) were found in Slovenian farmed freshwater fish samples. The results are not surprising regarding certain antibiotics such as spectinomycin, lincomycin and virginiamycin with withdrawal periods of five days (Cravedi 1990; Zimmermann and Büning-Pfaue 1993). No sample of freshwater fish was found with oxytetracycline residues, despite the long withdrawal period needed for the elimination of the drug depending on water temperature (Jacobsen 1989).

We also found no positive samples of chloramphenicol. Its use is banned in food-producing animals including fish because it is classified among the substances in Annex IV of the Council Regulation (EEC) No 2377/90 (Off J Eur Commun 1990), on the grounds of consumer safety. Because of its possible illegal use, mostly for its efficiency in treating various fish diseases, the effective monitoring of residues in food of animal origin, including farmed fish, is essential with very low limits of detection.

We detected sulphonamides in five samples (Table 3); all were trout taken from different fish farms. Only one sample, which contained 130 ng/g of sulphamerazine, exceeded the MRL level of 100 ng/g. We investigated its source

and determined that it originated in the feed. The farmer had used feed containing undisclosed sulphamerazine and unknowingly introduced sulphonamide into the food chain. Unfortunately, the fish farm was connected by water with another, where we also detected sulphamerazine in one fish sample. The concentration of sulphamerazine found in rainbow trout from the second farm was considerably lower (30 ng/g). The use of sulphonamides should be restricted on fish farms and efforts be made to avoid contamination of adjacent farms through connecting waterways. Withdrawal periods also differ for each drug: 12 days for sulphadiazine at 16° C (Intorre et al. 1996), but 8 days for sulphadimidine at 12° C (Haagsma et al. 1990).

Table 3. Concentration and type of sulphonamides found in trout muscle/skin tissue.

Type of sulphonamide	Content ng/g
sulphamerazine	130
sulphamerazine	30
sulphamonomethoxine	30
sulphamonomethoxine	30
sulphamethoxazole	10

Acknowledgments. Financial support for this work was provided by the Ministry of Science and Technology and the Ministry of Agriculture, Forestry and Food of the Republic of Slovenia (L4-0771).

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