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DRUG WITHDRAWAL FROM FARMED FISH*

DEPLETION OF OXYTETRACYCYLINE, SULFADIAZINE, AND TRIMETHOPHRIM FROM MUSCULAR TISSUE OF RAINBOW TROUT (SALMO GAIRDNERI)

By

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SALTE, RAGNAR and KNUT LIESTØL: *Drug withdrawal from farmed fish. Depletion of oxytetracycline, sulfadiazine, and trimethoprim from muscular tissue of rainbow trout (Salmo gairdneri).* Acta vet. scand. 1983, *24*, 418—430. — A statistical approach to the setting of withdrawal times is presented. It is suggested that the time intercept between detection limit of the applied method of analysis, and the 90 % upper prediction limit of a linear regression on the logarithm of the tissue drug concentration provides a realistic estimate of the necessary withdrawal period at the temperatures considered. Recognizing that water temperature is an important determinant of pharmaco-kinetics in fish, temperature-dependent withholding periods are recommended, i.e. 60 days at temperatures above 10°C and, for oxytetracycline, 100 days at temperatures between 7 and 10°C. The observed persistence of sulfadiazine and trimethoprim at lower water temperatures indicates that this drug mixture should be applied to slaughter fish only during summer.

medicated feed; oxytetracycline; sulfadiazine; trimethoprim; elimination; residues; muscle; rainbow trout; Salmo gairdneri.

The widespread use of antibiotics and chemotherapeutic drugs in modern, intensive aquaculture is well recognized, but inherent in this application of drugs to food-producing species is the public health aspect associated with tissue residue levels. Thus, drug withdrawal periods are required.

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A review of the relevant literature revealed few and vague recommendations concerning withdrawal times for fish treated with antibiotics or chemotherapeutic drugs. Working with chloramphenicol, *Utne Skaare et al.* (1974) concluded that a 14-day withholding period was sufficient at 6—14°C water temperature. *Dalgaard-Mikkelsen & Rasmussen* (1964) recommended a withdrawal over a period of 4 weeks in summer and over at least 6 weeks in winter for fish fed sulfamerazine in the diet, while *McCracken et al.* (1976), examining the depletion of oxytetracycline and trimethoprim, postulated that a withholding time of 3—4 months in winter would be necessary to meet public health standards.

Based on preliminary investigations into the elimination of oxytetracycline in rainbow trout (Salmo gairdneri) fed a commercial medicated feed (*Salte* 1982), the Norwegian Ministry of Agriculture tentatively recommended withdrawal periods of 40 days at water temperatures above 9°C and of 80 days at water temperatures below 9°C (*Landbruksdepartementet* 1982).

The concentration of drugs in body tissues changes over time in a way which may be well approximated by sums of exponential functions (*Baggot* 1977). The present study was designed to decide whether such approximations were adequate when applied to the time interval of post-distribution equilibrium, and, if so, to apply a statistical approach to the setting of withdrawal times based on quantitative analyses of drug residues in fish muscle.

MATERIALS AND METHODS

Materials

Six groups of healthy rainbow trout bred at the Research Station for Salmonids, Sunndalsøra Unit, served as test fish. All groups were held in running polyhaline water (salinity 25 ‰). Groups 1 and 4 (Table 1) were kept at ambient temperature in 75 m^2 circular, outdoor, concrete ponds with central drainage. Water level was maintained at approximately 60 cm with a flow of 250 l per min. For groups 2,3,5 and 6, 1 m^2 fibreglass tanks with chamfered corners and central drainage were used. Tanks were situated in a rearing hall where artificial light prevailed 24 h a day. Water temperature was controlled to within 1°C of the mean, and water levels were maintained at approximately 40 cm with a flow of 10 l per min.

Table 1. Dosage levels and feeding rates in relation to water temperature.

Group number	Number of fish/group	Weight (g) a	Water temperature (°C) b	Treatment	Dosage level (mg/kg of fish/day)	Feeding rate (% of body weight/day)	Experimental period (days) c
1	50	3,000	8.2±2.8	Oxytetracycline	37.5	3	40
2	100	60	7.5±0.4	Oxytetracycline	75	1	100
3	100	100	9.6±0.7	Oxytetracycline	75	4	40
4	50	3,000	8.1±2.9	Sulfadiazine-trimethoprim (5:1)	15	3	40
5	100	60	7.6±0.4	Sulfadiazine-trimethoprim (5:1)	30	1	100
6	100	100	9.7±0.7	Sulfadiazine-trimethoprim (5:1)	30	4	40

[a] Approximate average weight at the commencement of experiment
[b] Mean ± s
[c] Including the 10-day medication period

The test fish were allowed to adapt to the experimental conditions for at least 10 days, and all fish were starved for 2 days prior to treatment. Groups 1—3 received oxytetracycline (TMQ-oxytetracycline base, Pfizer), and groups 4—6 received sulfadiazine-trimethoprim (Tribrissen®, Wellcome) in commercial pellets (Skretting) for 10 consecutive days. Fish were fed by hand from 7^{30} a.m. until 3^{00} p.m. Dosages and feeding rates were as listed in Table 1, assuming that all the feed was consumed and drug leakage was negligible.

Sampling scheme and assay methods

Fish were invariably sampled at 5^{00} p.m. Three fish from each group were killed at intervals after treatment. Controls were included. Fish were stored at —20°C and assayed individually within 2 months of storage.

Muscle samples were taken from the dorso-lateral body area just posterior to the operculum. Samples were homogenized with the appropriate extractant. The fluid phase was then vacuum concentrated and resuspended in buffer (Table 2).

Extracts were assayed employing microbiological methods (Table 3). Inhibition zones were measured with callipers. Zones smaller than 3 mm were ignored. Sensitivities of the applied

Table 2. Extraction procedures for oxytetracycline and sulfadiazine-trimethoprim residues in fish muscle.

Antibiotic or chemotherapeutic drug	Extractant	Buffer for resuspension of extract	pH of buffer
Oxytetracycline	Methanol/ Hydrochloric acid 0.1 M (98 + 2)	Phosphate buffer	4.5±0.1
Sulfadiazine-trimethoprim	Ethyl acetate/ Sodium hydroxide 1 M (9 + 1)	Phosphate buffer	7.7±0.1

methods were calculated to be 0.04 µg/g, for oxytetracycline 0.04 µg/g for sulfadiazine, and 0.002 µg/g for trimethoprim.

To confirm the presence of active drug residues, extracts were submitted to chemical inactivation procedures (*Salte* 1983).

Statistical methods

For each series of experiments, it was evaluated if a sum of exponential functions provided a fair fit to the drug elimination data. The parameters were estimated either by nonlinear least-square regression (applying the Marquardt method available in the Statistical Analysis System (SAS-procedure NLIN)), or, when one exponential was sufficient, by linear regression on the logarithm (ln) of the drug residue concentration (SAS-procedure GLM). Confidence limits and prediction limits (i.e. limits which will include a future observation with a certain probability) were estimated from the linear regression employing the common methods based on Gaussian distributed residuals with equal variance (see e.g. *Sachs* 1982). In the late elimination phase

Table 3. Microbiological methods for the detection of oxytetracycline, sulfadiazine, and trimethoprim in extracts from fish muscle.

Antibiotic or chemotherapeutic drug	Test medium	pH of medium	Test organism	Preincubation -time(h)	Preincubation -temp (°C)	Incubation -time(h)	Incubation -temp (°C)
Oxytetracycline	Antibiotic Medium 8 (Difco 667)	5.9±0.1	*Bacillus cereus var. mycoides* ATCC 11778	2	4	16—18	30
Sulfadiazine	Standard II Nähragar (Merck 7883)	6.0±0.1	*Bacillus subtilis* ATCC 6633	1	20	18—20	30
Trimethoprim	DST agar (Oxoid CM 261)	7.4±0.1	*Bacillus pumilus* CN 607	½	20	18—20	30

residue levels sometimes were below the lowest detectable concentration. Values were then assigned so that the non-linear and the linear procedures provided compatible estimates.

No separation of age, sex, maturity effects or tank/pond effects were attempted in the analyses, since insufficient data were available.

Weight of fish was considered as a potential determinant of the pharmacokinetics, and weight was therefore entered into the regression equations as a covariate (groups 1 and 4).

The figures were constructed by means of the graphical procedures available in SAS.

RESULTS

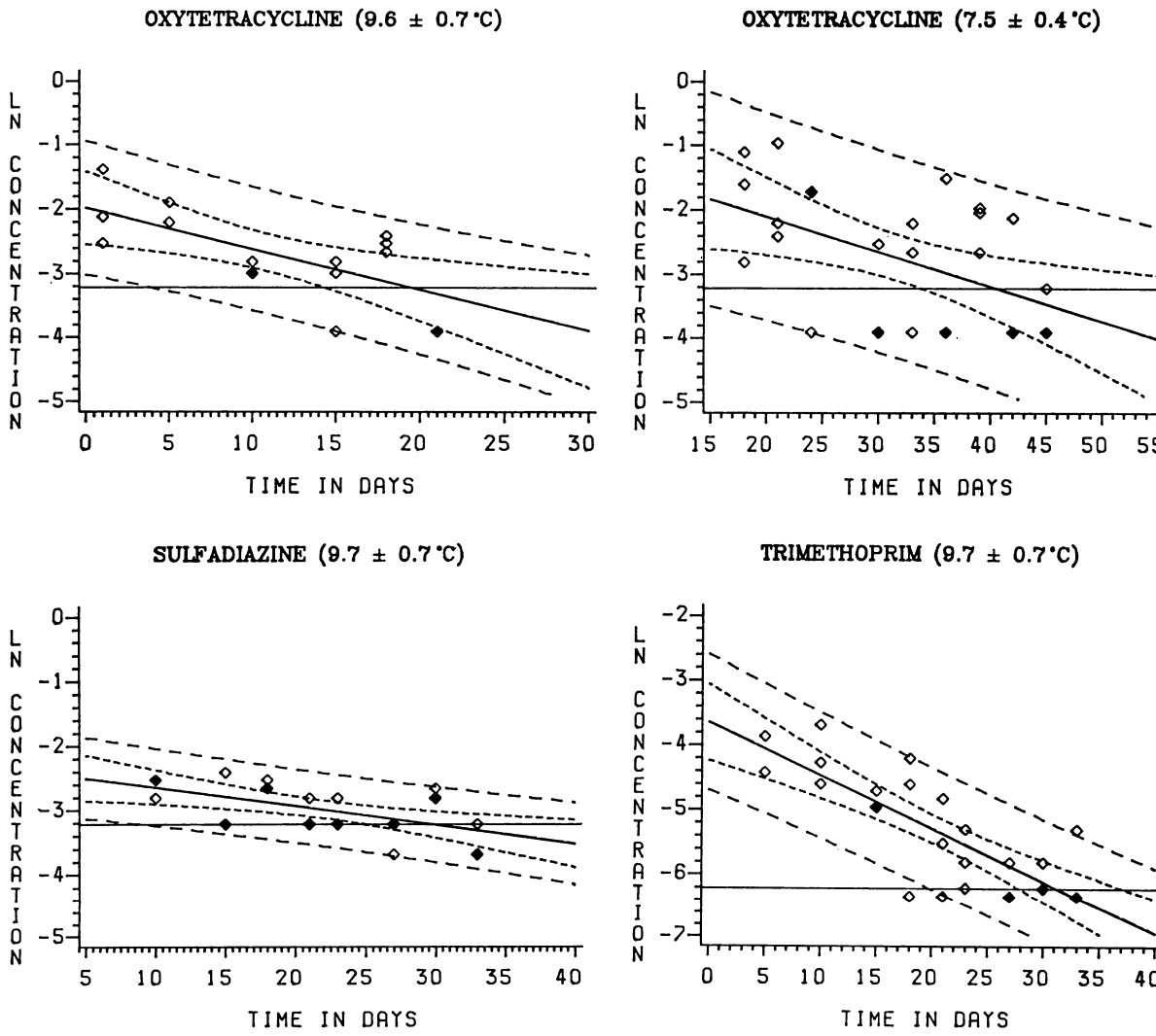
There was a considerable fish-to-fish variation in drug residue levels within each group at any post treatment sampling time.

A one-exponential approximation usually proved to be adequate as a means of describing the tissue drug concentration versus time relationship of the elimination phase. Furthermore, drug depletion from muscular tissue was closely related to water temperature, which is clearly demonstrated in the variation of the apparent post-distribution phase elimination rate constant (β).

For oxytetracycline the 9.6±0.7°C water temperature β-value (Group 3) was estimated to be 0.069± 0.021 day (± s.e.), while the 7.5±0.4°C value (Group 2) was estimated to be 0.056±0.019. Accordingly, the per 1°C decrease increase in β-value approximated 10 %.

At 9.6±0.7°C residual tissue levels of oxytetracycline were estimated to fall below 0.04 µg/g, with a probability of 95 %, at about 40 days post treatment, referring to the estimated time intercept between detection limit of the applied assay method and the extrapolated 90 % upper prediction limit of a plot of ln concentration against time (Fig. 1a). The corresponding 7.5± 0.4°C time intercept was estimated to be at about 90 days post treatment (Fig. 1b).

For sulfadiazine the 9.7±0.7°C β-value (Group 6) was estimated to be 0.029±0.009, and residual tissue levels were estimated to fall below 0.04 µg/g, with a probability of 95 %, after about 60 days (Fig. 1c). A two-exponential model applied on the same



Figures 1a—1d. Computer diagrams summarizing the functional relationship between the logarithm (ln) of the drug concentration (◇) in fish muscle and time, during the post-distribution elimination phase; (a and b) oxytetracycline, (c) sulfadiazine, (d) trimethoprim. Outer broken lines are 90 % prediction limits and inner broken lines are 95 % confidence limits to regression estimates for the data. Solid horizontal line is detection limit of the applied method of analysis. Filled diamonds (◆) represent coinciding observations. Note differences in time-scale.

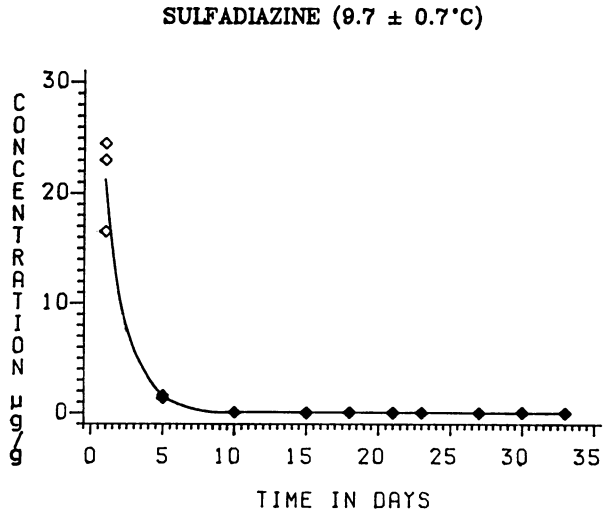


Figure 2. Concentration of sulfadiazine (◇) in fish muscle during distribution and elimination phases. Two-exponential model.

data, but including observations made at 1 and 5 days post treatment, revealed an initial steep decrease (estimated distribution constant 0.67±0.19) in residue levels terminating in a nearly horizontal line after a mere 10—12 days (Fig. 2). A 7.6±0.4°C time intercept for residual sulfadiazine levels defied estimation, the post-distribution elimination rate constant not being significantly different from zero ($P = 0.88$).

The trimethoprim apparent elimination rate constants were estimated to be 0.079±0.014 (9.7±0.7°C, group 6) and 0.026± 0.014 (7.6±0.4°C, group 5). The elimination of trimethoprim proved to follow a pattern similar to that of sulfadiazine, however, with a significant decrease of the small but persistent remainder at both temperatures ($P < 0.05$).

At 9.7±0.7°C residues of trimethoprim were estimated to fall below 0.002 µg/g of muscle tissue after about 45 days (Fig. 1d), while the corresponding 7.6±0.4°C time intercept would occur after more than 200 days post treatment, hereby indicating the uncertainty involved in estimating this time intercept.

Weight of fish (range 1.8—4.4 kg, groups 1 and 4) had only a minor effect on the values of the post-distribution elimination rate constant.

DISCUSSION

Data obtained in conventional residue protocols show pronounced animal-to-animal variation in tissue residue levels, as illustrated by the rather wide confidence intervals in the plots of the logarithm of the concentration against time. This variation is determined mainly by the total medicated feed intake, the stomach emptying rate, the absorption of the drugs, and the performance of the fish in terms of their metabolic and excretory capability in handling the drugs;— factors which are all temperature - dependent in poikilothermic species (see e.g. *Burrows* 1972, *Windell et al.* 1976, *Baggot* 1977, *Brett & Glass* 1973).

Systematic variation attributable to breed, age, sex and maturity effects within the experimental groups, and to tank or pond effects between groups will further contribute to the scattering of the data. Finally, variation due to the analytical procedures must be considered.

The regression on tissue residue levels generally revealed a fair approximation of the drug concentration versus time relationship of the post-distribution elimination phase of the considered drugs.

In the later part of the elimination phase, the tissue drug concentrations were sometimes below the lowest detectable value. When using the classical procedure of linear regression on log concentration, the values assigned to these observations had a pronounced effect on the estimates of the parameters (the log transformation "blows up" small differences close to zero). These missing values were chosen so that estimates obtained from the linear regression on log concentrations and the estimates obtained from the non-linear regression were consistent. The observed log values were then fairly equally scattered around the regression line throughout the considered time interval, that is, the distribution of the residuals was approximately equal at all times considered. This distribution appeared to be fairly normal. Thus, confidence bands were estimated employing methods based on Gaussian distributed residuals with equal variance.

Estimates of the apparent elimination rate constants (β) of the post-distribution phase were based on the assumption that post-distribution equilibrium was established at 5 and 18 days post treatment at 9—10°C and at 7—8°C water temperature, respectively. (Fig. 1a, however, includes observations of day 1 post treatment. While having a minor influence on the estimated

β-value, this improved the determination of the confidence bands). Moreover, the elimination processes were assumed to follow first order kinetics. Once distribution equilibrium has occurred the elimination rate constants of different tissues will remain equal and constant (see e.g. *Dahl* 1974, *Mercer et al.* 1977), supporting the feasibility of this approach.

The tissue drug concentration approaches zero asymptotically. Consequently, one cannot predict the time at which all drug would have been removed from the tissues. Furthermore, it is only conceivable to detect residual tissue levels at or above the detection limit of the applied method of analysis. While recognizing the uncertainty applying to estimates which are based on extrapolations, it is, however, possible to predict the time required for the tissue drug concentration to reach a desired level. Since there is no unique approach to the setting of withdrawal times, the time intercept between detection limit and the 90 % upper prediction limit was therefore considered to give a realistic estimate of the necessary withdrawal periods for the relevant temperature intervals.

The temperature dependence of oxytetracycline elimination rate was according to expectations, i.e. a near 10 % decrease or increase per 1 °C change in water temperature (*Ellis et al.* 1978). The elimination of sulfadiazine and trimethoprim, however, appeared to disagree somewhat with these expectations, the estimated β-values of the cold water groups being considerably lower than would have been expected from the warm water group estimates. A probable explanation of this apparent inconsistency is that the elimination processes shortly reach a point after which there is only a small but persistent remainder in the tisssues. Moreover, due to drug build-up during the treatment period (*McCracken et al.* 1976) this remainder would be likely to level off at a higher concentration at lower water temperatures, so facilitating the detection of significant residue levels for a longer period of time in the cold water groups (i.e. up to 90 days after end of treatment). The present results compare favorably with these assumptions, and are consistent with the findings of *Cartmell et al.* (1976) in respect of trimethoprim residues.

The present results further suggest that sulfadiazine is the more potent residue producer of the drug mixture, which is contrary to the essentially complete elimination of sulfadiazine reported by *Cartmell et all.* (1976). In support of the present

findings the pharmacokinetic profiles of both sulfonamide and trimethoprim will be closely matched to give a constant ratio of 20:1 in body fluids and tissues (*McCarthy et al.* 1974). Even if this ratio in tissues is frequently less than 20:1 (*Mandell & Sande* 1980) the concentration of sulfonamide at a given time will far exceed that of trimethoprim, as illustrated by the 10-15:1 ratio of sulfadiazine to trimethoprim demonstrated in the present study. Moreover, being a weak electrolyte with pKa 6.5, sulfadiazine will probably be bound to different tissues to a greater extent than trimethoprim (pKa 7.6) at physiological pH. Since tissue binding is generally reversible (*Mayer et al.* 1980), it may be postulated that the depletion of trimethoprim proceeds at a higher rate than that of sulfadiazine.

Weight of fish did not affect the regression on residue concentrations when tentatively entered as a co-variate, thus suggesting a weight independence of the drug elimination rate under the conditions present. In support of this suggestion are the findings of *Brett & Glass* (1973) stating that the metabolic rate of sockeye salmon (Oncorhynchus nerka) was virtually independent of size at all temperatures.

CONCLUDING REMARKS

Water temperature is an important determinant of the pharmacokinetics in fish. Thus, temperature-dependent drug withdrawal periods must be established and also become criteria for usage.

Lower doses can comply with withdrawal periods established at a high dose, as can dosage schedules of less duration than the ones applied. Estimates cannot, however, be made to other routes of administration or to other formulations of the drugs. Likewise, it is not conceivable to make extrapolations to other water temperatures for the same dose and duration of treatment, even if estimates of β-values (based on a 10 % decrease/increase per 1°C change in water temperature) may be valid. This is because the present experimental design provides no specific data base on the absorption and distribution of the drugs, so precluding an estimation of any time intercept at other water temperatures.

Given the above restrictions, the following can be recommended:

For fish treated with oxytetracycline at a dose of up to 75 mg

per kg of fish and day for not more than 10 consecutive days, a withholding period of 60 days at water temperatures above 10°C, and of 100 days at water temperatures between 7 an 10°C.

For fish treated with sulfadiazine-trimethoprim at a dose of up to 30 mg per kg of fish and day for not more than 10 consecutive days, a withholding period of 60 days at water temperatures above 10°C.

The sulfadiazine-trimethoprim results indicate that this drug mixture should be applied to slaughter fish only during summer, i.e. at water temperatures of about 10°C or more. This does not, however, exclude the recognition of a sulfadiazine-trimethoprim potential in the medication of smaller fish around the year.

Groups of fish to be slaughtered within the recommended withholding periods should be submitted to analyses comprising duplicate samples from not less than 5 randomly selected individuals. Analyses should be performed during the compulsory 7-day pre-slaughtering starvation period (*Fiskeridepartementet* 1981), and slaughtering should not be permitted unless all samples are negative. (It should be noted that the above number of samples is a rough estimate based on what it is considered possible to accomplish for routine purposes. To be 80 % sure of detecting a positive reagent if 5 % of the fish contained detectable residues of the drug in question, it would be necessary to analyze not less than 32 fish).

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SAMMENDRAG

Tilbakeholdelse ved behandling av oppdrettsfisk. Utskilling av oxytetracyklin, sulfadiazin og trimethoprim fra muskelvev hos regnbueørret (Salmo gairdneri).

Det presenteres en statistisk metode for bestemmelse av nødvendige tilbakeholdelsesfrister. Skjæringspunktet mellom analysemetodens følsomhet og den øvre grense for et 90 % konfidensintervall for enkeltobservasjoner for den lineære regresjonsfunksjon som beskriver sammenhengen mellom logaritmen av vevskonsentrasjon og tid, ansees å gi et realistisk anslag på nødvendig tilbakeholdelser under de gitte temperaturforhold. På grunn av vanntemperaturens betydning for utskilling av farmaka hos fisk, anbefales temperaturavhengige tilbakeholdelsesfrister. Etter behandling med oxytetracyklin innebærer dette en tilbakeholdelsesfrist på 60 døgn ved vanntemperaturer over 10°C og 100 døgn ved tempearturer mellom 7 og 10°C. Det anbefales videre at kombinasjonen sulfadiazin-trimethoprim anvendes til slaktefisk bare i sommerhalvåret.

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