PHARMACOKINETICS AND BIOAVAILABILITY OF OXYTETRACYCLINE IN ATLANTIC SALMON HELD IN SEAWATER AT DIFFERENT TEMPERATURES

A Thesis

Submitted to the Graduate Faculty

in Partial Fulfilment of the Requirements

for the Degree of

Master of Science

in the Department of Pathology and Microbiology

Faculty of Veterinary Medicine

University of Prince Edward Island

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Charlottetown, P.E.I.

December, 1992

ISBN 0-315-81260-5

Canada

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ABSTRACT

Oxytetracycline (OTC), a broad spectrum antimicrobial with considerable activity against Gram-negative organisms, is one of the most commonly used antibacterials in aquaculture. Pharmacokinetic data is limited in fish. Studies to determine the optimum treatment conditions and withdrawal time for fish in saltwater indicate differences from the data available for fish in fresh water. There are no studies which summarize the plasma pharmacokinetics and bioavailability of oxytetracycline in Atlantic salmon (*Salmo salar*), in seawater, or at different temperatures.

The Association of Official Analytical Chemists (AOAC) microbiological assay of oxytetracycline in feeds was modified to a well-diffusion assay and successfully adapted for the determination of oxytetracycline in fish plasma. Handling and storage conditions of plasma samples were evaluated. Aliquots of OTC spiked fish plasma were stored in glass and in polypropylene vials at - 70° C for seven weeks. The concentration of antibiotic was determined weekly. Statistical analysis indicated that no significant reduction of OTC in fish plasma had occurred in either the glass ($p = 0.696$) or the polypropylene ($p = 0.630$) vials over seven weeks. No significant difference was demonstrated between the use of glass or polypropylene vials ($p = 0.893$) as storage containers for fish plasma containing OTC.

A pilot study was undertaken to evaluate the suitability of sampling times, form of oral dose, and trends in the plasma pharmacokinetics of oxytetracycline. The pilot study consisted of groups of ten Atlantic salmon held in reconstituted seawater at 10° C treated with OTC at two concentrations, 100 mg and 200 mg OTC/kg body weight, either as a single intraperitoneal injection, or as a single oral dose via a gelatin capsule or in a 0.2% w/v aqueous Bacto-Agar slurry administered by stomach intubation. Gelatin capsules were slow to hydrolyze. Consequently, of the two methods for administration of OTC via the oral route, the aqueous Bacto-agar slurry was the preferred method. Since the elimination phase had not commenced by the end of the sampling time of 36 h, it was not possible to draw any conclusions about the pharmacokinetics of OTC elimination.

To study the plasma pharmacokinetics of OTC in Atlantic salmon held in seawater, groups of eight Atlantic salmon of an average weight of 470.1 ± 39.9 g (mean ± 1SD), were randomly assigned to two treatment groups at 10° C or at 15° C. Cannulation of the dorsal aorta was performed. Oxytetracycline was administered, at the rate of 100 mg/kg body weight, via intra-aortic (IA) injection through the catheter to five fish at each temperature, and per os (PO) via intubation

to five fish at each temperature. Plasma samples, collected at predetermined times over an 8 day period, were assayed using the modified AOAC microbiological assay for oxytetracycline in feeds. Pharmacokinetic parameters including bioavailability (BA), elimination half-life ($T_{1/2}$), maximum concentration of drug (C_{max}), time to maximum plasma concentration (T_{max}), plasma clearance (Cl) and volume of distribution at steady-state (VD_{SS}) were determined. The results indicated that bioavailability was 1.5% in Atlantic salmon held in reconstituted seawater at 10° C and 7.4% for Atlantic salmon at 15 ° C. A General Linear Model was used for statistical analysis and is indicated that the route of administration significantly influenced all pharmacokinetic parameters, while temperature was significant only for BA. A factorial analysis indicated that there were simple temperature effects on C_{max}, T_{max}, VD_{SS} and $T_{1/2}$. There was no influence of temperature on plasma clearance. Over the 196 h study period, oxytetracycline was eliminated from Atlantic salmon held at 10° C by a first order process best described by an open three-compartment model. At 15° C elimination of oxytetracycline was best described by an open two-compartment model.

The low apparent bioavailability of oxytetracycline in Atlantic salmon held in seawater at 10° C and 15° C described in this study supports earlier accounts of low bioavailability of oxytetracycline in teleosts.

ACKNOWLEDGEMENTS

Research is never a solitary pursuit, but rather occurs within a framework of discussion and technical support. I thank the members of my supervisory committee Dr. Ray Long, Dr. Andrew Tasker, Dr. Geoff Rawling, Dr. Don Rainnie and Dr. Gerald Johnson for their helpful discussions and support throughout this study. I especially want to thank Dr. Els Cawthorn, who was the project surgeon throughout this study, and Grant Curtis for assisting in the care and feeding of the Atlantic salmon, and for his assistance during the surgeries. I would also thank the other members of the surgical teams for all of their assistance: Lee Dawson, Paul Lyon, Dr. Maura Jansen, Dr. Larry Hammell, and Denise Dorian.

Finally, I would also like to thank my husband, Ivan MacSwain, and our five children for their support and understanding over the past two years.

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NOTATION

AUC	area under concentration vs time curve
β	beta
BA	bioavailability
° C	degrees celsius
CI	confidence interval
Cl	plasma clearance
C_{max}	maximum serum concentration
γ	gamma
GER	gastric evacuation rate
g	gram
HPLC	high-pressure liquid chromatography
h	hour
IA	intra-arterial
IE	intra-oesophageal
IP	intraperitoneal
kg	kilogram
L	litre
mg	milligram
mL	millilitre
PO	per os (oral)
r^2	correlation coefficient

SD	standard deviation
$T_{1/2}$	half-life of elimination
T_{max}	time to maximum serum concentration
VD_{SS}	volume of distribution at steady state
µg	microgram
%	percentage
±	plus or minus
≤	less than or equal to

1.0 GENERAL INTRODUCTION

1.1 Introduction

The beginning of aquaculture, or fish farming, is unknown. However, approximately 2000 BC, there is evidence that the Chinese were fish farming and the Japanese were involved in oyster culture. By the Middle Ages, aquaculture was well established in both European and Asian countries, but it was not until 1857 that aquaculture (salmonids) began in North America (16). Initially, the industry developed slowly. After World War II aquaculture underwent rapid growth, stimulated by innovative technologies which led to the development of a faster-growing, rapidly processed and superior product for the marketplace.

There is concern that the world is approaching a plateau in the capture fisheries, but the world population continues to grow and with it the demand on food resources. Aquaculture will be expected to assume an increasingly important role in the production of the world's food supply. Data from the Food and Agricultural Organization of the United Nations (1989) shows that aquaculture accounted for a total of 11.1% of the total fisheries production, an increase of 3.2% since 1984 (64). With further development and new techniques, there is a lot of room for expansion in the aquaculture industry. The potential for more frequent disease episodes will also be increased with enhanced intensive production.

1.1.1 Use of Chemotherapeutics in Aquaculture: Modern aquaculture facilities rely on stocking fish at high densities in order to be competitive on the international market. The confinement of large numbers of salmon in sea cages leaves the fish vulnerable to pathogens or contaminants which may be present in the water column. Good husbandry practices will minimize the risk of disease, however if a disease outbreak should occur, the high densities at which the fish are maintained will have a tendency to aggravate morbidity and mortality, with potentially devastating economic results. To counteract disease episodes and reduce economic losses, aquaculturists have come to rely on the use of chemotherapeutics.

Fish diseases are caused by a wide range of infectious agents including viruses, bacteria, fungi, protozoan and metazoan parasites. Bacterial infections frequently cause major finfish losses (5). Potential fish pathogens have been identified from some 21 bacterial Genera (5). It was not until the late 1930s that efforts commenced to control systemic bacterial diseases with chemotherapeutics (1). As soon as suitable drugs (human or veterinary) became available they were tested for the control of fish disease. Despite these efforts, the number of chemotherapeutics available tc the aquaculturist remains limited. In the 1950s oxytetracycline (OTC) became the antibiotic of choice (16, 18). The use of antibiotics introduces several concerns including the potential for environmental loading with chemotherapeutics which could lead to the selection of bacteria which are resistant, and public health considerations, specifically tissue residues levels.

1.1.2 Oxytetracycline, Structure and Mode of Action: Tetracyclines are produced by *Streptomyces* spp. of fungi. Oxytetracycline (Figure 1), a broad spectrum antibiotic with considerable activity against Gram-negative bacteria, is bacteriostatic. The antibiotic inhibits the synthesis of proteins by binding to bacterial 30S ribosomal subunits (19, 73).

The earliest investigations into the potential for oxytetracycline use in aquaculture occurred in 1951, when it was shown to be effective against ulcer disease, caused by *Haemophilus piscium*, in brook trout (*Salvelinus fontinalis*) (78). Since that time it has become one of the most frequently used antibiotics in aquaculture. Oxytetracycline was approved for use in food fishes in Canada, in 1989. Throughout the aquaculture industry, oxytetracycline is administered at a dose rate of 75 - 125 mg OTC/kg body weight/day in feed for ten days.

1.2 Pharmacokinetics of Oxytetracycline in Fish

The pharmacokinetics of a chemotherapeutic are a function of the absorption, distribution, transformation and excretion rates of the drug and its metabolites. In poikilothermic species, such as fish, all of these parameters are temperature dependent (12). A change of 1° C in water temperature will result in the alteration of metabolic rate by approximately 10% (26). The increased or decreased rates of absorption, distribution, transformation and elimination will be reflected in changes

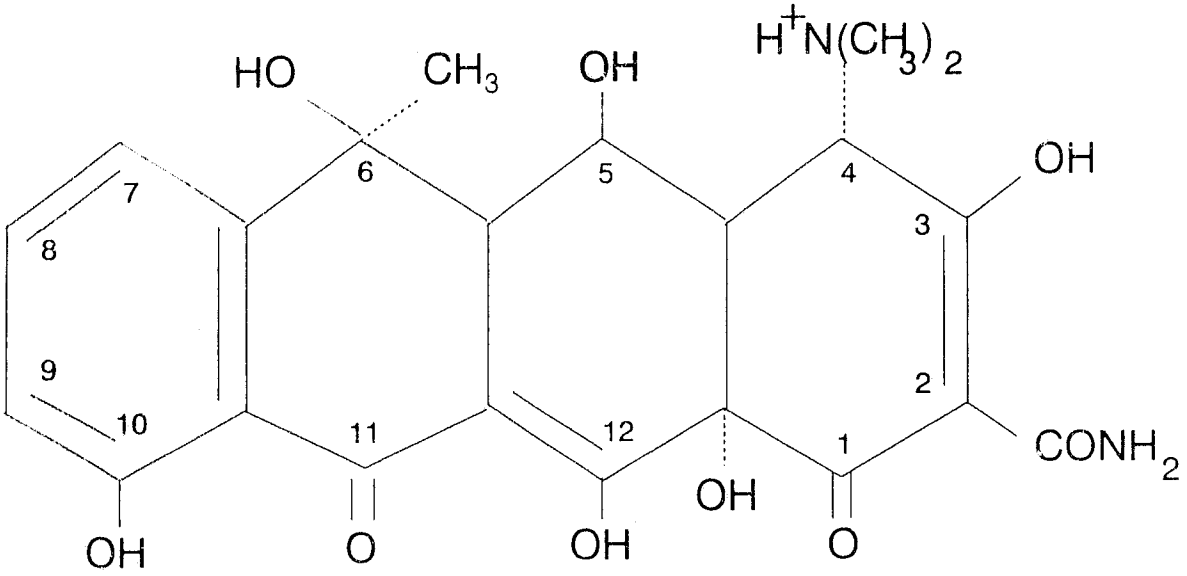

Figure 1. Structure of oxytetracycline

in the tissue concentrations of the drug and its metabolites (1, 12, 26, 48, 74). Pharmacokinetic data is limited for fish and there are indications of apparent differences between fish species. Inappropriate administration of antimicrobials could result in toxic side effects such as immunosuppression, or tissue damage (34).

Currently, the most convenient and cost-effective method of treatment with antibiotics is to administer them as feed additives. As a delivery system this method has a number of disadvantages such as variability of dose received, resulting from differences in the amount of feed ingested, as well as variations between individuals in their ability to absorb the drug from the gastro-intestinal tract. Feeding competition, palatability (taste of the medicated feed) and disease status will also influence feed consumption. Other methods of antibiotic delivery (intra-vascular, intra-muscular, or intra-peritoneal injection) permit more certain and accurate dose delivery, but are labour intensive and stressful to the fish. They are rarely used except for valuable brood stock.

Drugs absorbed from the gastrointestinal tract, generally by passive diffusion, must pass through the liver before they reach systemic circulation. Hence, hepatic metabolism and excretion may significantly influence a drug's bioavailability. Bioavailability is the term used to indicate the extent to which a drug reaches systemic circulation intact. The bioavailabilities of several drugs, including the

tetracyclines, are dramatically lower in several fish species as compared to mammals (45).

Tetracyclines have a number of potential metal binding sites, and the ability of tetracyclines to form complexes with divalent and trivalent cations is well established. This ability is especially strong for hydrophillic tetracyclines, such as oxytetracycline, and results in complexes which are poorly absorbed compared to the free drug (43, 55, 57). In digestibility studies, Cravedi *et al.*(21), determined that the bioavailability of oxytetracycline in rainbow trout (*Oncorhynchus mykiss*) was in the 7-9% range. In human beings, the absorption for tetracycline is approximately 60%, however the percentage of absorption is directly related to the amount of calcium contained in the food or fluids ingested, at or near, the time of consumption. Most commercial fish feeds contain approximately 21.6 g total calcium/kg of feed (twenty times the amount found in human diets) (21). While the total calcium present in the feed does not accurately represent the potential for binding oxytetracycline, the larger the quantity of calcium, the more likely that the bioavailability of oxytetracycline will be reduced through chelation with the calcium in the feed.

Pharmacokinetics and bioavailability of oxytetracycline following intravascular and oral administration have been investigated in carp (*Cyprinus carpio* L.) (33), African catfish (*Clarias gariepinus*) (35), channel catfish (*Ictalurus punctatus*) (69) and rainbow trout (*Oncorhynchus mykiss*) (13, 35). The tissue distribution of ^{3}H-

tetracycline in rainbow trout was studied using whole-body autoradiography (46). Residue studies following oral administrations of oxytetracycline have been undertaken in rainbow trout (13, 18, 48, 56, 65, 74). There are no studies which summarize the plasma pharmacokinetics and bioavailability of oxytetracycline in Atlantic salmon (*Salmo salar*), in seawater, or at different temperatures.

1.3 Research Objectives

The primary research objectives described in this thesis were threefold:

1) To adapt and standardize the AOAC microbiological method for the assay of oxytetracycline in feed (84) for the measurement of oxytetracycline concentrations in the plasma of Atlantic salmon.

2) To determine the plasma pharmacokinetics of oxytetracycline in Atlantic salmon held in reconstituted seawater at 10° C and at 15° C, following intravascular and oral administration.

3) To determine the apparent oral bioavailability of oxytetracycline in Atlantic salmon held in seawater at 10° C and 15° C.

2.0 STANDARDIZATION OF MICROBIOLOGICAL ASSAY FOR OXYTETRACYCLINE IN THE PLASMA OF ATLANTIC SALMON

2.1 Introduction

The quantitative assay of large numbers of plasma samples requires a sensitive, accurate and preferably rapid method of analysis. Microbiological assays (4, 10, 18, 39, 59, 74, 84), liquid chromatography (LC) techniques (82), and high-pressure liquid chromatography (HPLC) methods (11, 27, 47, 56, 63, 65) have been described for the analysis of oxytetracycline in body fluids and tissues. The choice of method will be a compromise based on a critical evaluation of the method characteristics required, as well as the availability of resources.

High pressure liquid chromatography (HPLC) is relatively rapid, specific, and sensitive, but it is expensive in terms of materials and equipment (24). The evaluation of chemotherapeutics in fish plasma usually involves a lengthy sample clean-up and pre-treatment of the plasma which may preclude the use of HPLC for routine analysis. Similar difficulties exist for the use of liquid chromatography (LC). Liquid chromatography methods are specific, but require time consuming extractions of oxytetracycline by heating in an acid solution followed by a deproteination step before the assay can be undertaken. Microbiological assays have been frequently used to measure antibiotic concentrations in various animal and plant tissues and

body fluids (20, 23, 24, 72). Such assays are inexpensive in terms of materials and equipment; simple, reliable and reproducible, when performed by experienced investigators; and can be used to test for a variety of agents with minimal revision (20, 24, 72). Disadvantages which have been reported for microbiological assays include the length of time required, low or variable specificity (11, 66, 69, 71), and vulnerability to operator biases (ie, the reading of zones of inhibition is not totally objective) (24, 49, 69). None the less, for the purposes of this study, the microbiological assay was selected for preliminary evaluation.

There are a number of properties which need to be considered, including accuracy, specificity, and sensitivity before choosing the type of microbiological assay.

2.2 Association of Official Analytical Chemists (AOAC) Microbiological Assay for Oxytetracycline in Feed

2.2.1 Introduction: The principle of a microbiological assay is that the diameter of the zone of inhibition is directly proportional to the amount of antibiotic in the sample. Assays are described as either two- or three-dimensional assays on the basis of antimicrobial diffusion from the source. In a two-dimensional assay, diffusion is directed against a seeded wall of bacteria, from either a well cut in the agar or a paper disc containing the antibiotic. This allows the antibiotic to be distributed equally throughout the agar layer. Three-dimensional assays use cylinders, fish spines

or disks containing the antimicrobial placed on the surface of a seeded agar plate. The antibiotic diffuses from the surface of the agar 180 degrees from its source. If variability exists in agar thickness then the antimicrobial will diffuse differentially. This will result in uneven zones of inhibition. The well-diffusion, two-dimensional assay, considered theoretically more reliable than the three-dimensional assay (24), was preferred over the paper disc two- or three-dimensional methods. There are several problems associated with the use of paper discs. First, because of the variability in the paper products, it is difficult to control the amount of drug which will actually be absorbed. Secondly, the paper may act as a chromatogram and separate the antimicrobial into individual components which may influence the size of the zone of inhibition. It is difficult to position paper horizontally onto the agar surface in order for the antimicrobial to diffuse into the surrounding agar in an uniform fashion. Edberg (24) suggests that well-type assays are 5 - 6 times more sensitive than techniques using paper disks, and in general this will allow the determination of lower concentrations of antibiotic in unknown samples.

2.2.2 Preparation of Plates for the AOAC Microbiological Assay: The Association of Analytical Chemists (AOAC) microbiological assay for oxytetracycline in feed (84) is a three-dimensional assay, utilizing stainless steel cylinders placed onto the surface of seeded agar to hold the test solutions. The AOAC method utilizes a large number of replicates for each standard solution and unknown sample. Cylinders are positioned onto the agar surface in a hexagonal pattern. Three alternate cylinders

are filled with a reference standard solution, while the remaining three cylinders are filled with the unknown solution. Plates are prepared, in triplicate, for each unknown. This will generate nine zones of inhibition for the reference standard solution and nine zones of inhibition for the unknown solution. Using a standard response curve (section 2.2.4) and a correction procedure (section 2.2.5) the concentration of the unknown can be determined. This is a rigorous method, but it is not cost-effective for the routine testing of small numbers of samples. To enhance the AOAC microbiological assay, modifications were introduced which would maintain the high degree of accuracy while converting it to the theoretically more sensitive two-dimensional well-diffusion assay.

2.2.3 Modification of the AOAC Method: Assay plates were prepared with 20 mL of Difco Antibiotic Medium #8 (pH 5.8 ± 0.3) per petri plate (15 x 100 mm) with an overlay of 5 mL Difco Penassay Medium (pH 6.6 ± 0.2) previously seeded with 10^5 (0.02 mL Difco spore suspension/ 100 mL Penassay medium) of the indicator organism, *Bacillus cereus* var. *mycoides* (American Type Culture Collection 11778). Instead of stainless steel cylinders, the assay plates were modified by removing agar plugs from the solidified agar plates with a 4 mm cork borer, cold sterilized by immersion in 70% alcohol for 24 hours (10), to generate a hexagonal pattern of wells. To seal the bottom of each well, aliquots of 20 µL of molten Antibiotic Medium #8 were dispensed into each well with a micropipettor.

2.2.4 Standard Response Curve: Oxytetracycline hydrochloride, obtained from Pfizer Canada Ltd., was dissolved in 0.1 N HCL and diluted in fish plasma to prepare standard solutions with final concentrations of 0.05, 0.10, 0.20, 0.25, 0.5, 1.0, 2.0, 4.0, 8.0 μg OTC/mL. The reference standard concentration was 0.2 μg OTC/mL. Using the modified AOAC method (see section 2.2.3) three alternate wells of the assay plates were inoculated with 20 μL of a standard solution and the remaining three wells receive 20 μL of the reference standard solution. The volume of plasma filled the wells to a convex meniscus. Three replicate plates were used for each standard solution. The plates were incubated at 37° C for 24 h, at which time diameters of the zones of inhibition, including the well diameter, were measured (to the nearest 0.05 mm) with dial callipers. Using the AOAC correction procedure (see Section 2.2.5) and the computer program, Minitab (Minitab Statistical Package, Release 7.1) (62), to perform least square regression analysis, the mean inhibition zone (mm) was plotted against the $\log_{10}$ OTC concentration (μg/mL) to produce a standard response curve (Figure 2). Over the course of the studies, the limit of detection was 0.05 μg OTC/ml plasma and the limit of quantification was 0.1 μg OTC/ml plasma.

2.2.5 AOAC Method of Mathematical Analysis: In each set of three plates, nine measurements of the zones of inhibition for both the reference standard and the standard solution were recorded. The average of the inhibition zone diameter of 36 wells for the reference standard solution (0.2 μg/mL) from 12 plates was the correction point for the standard response curve. A corrected zone of inhibition was

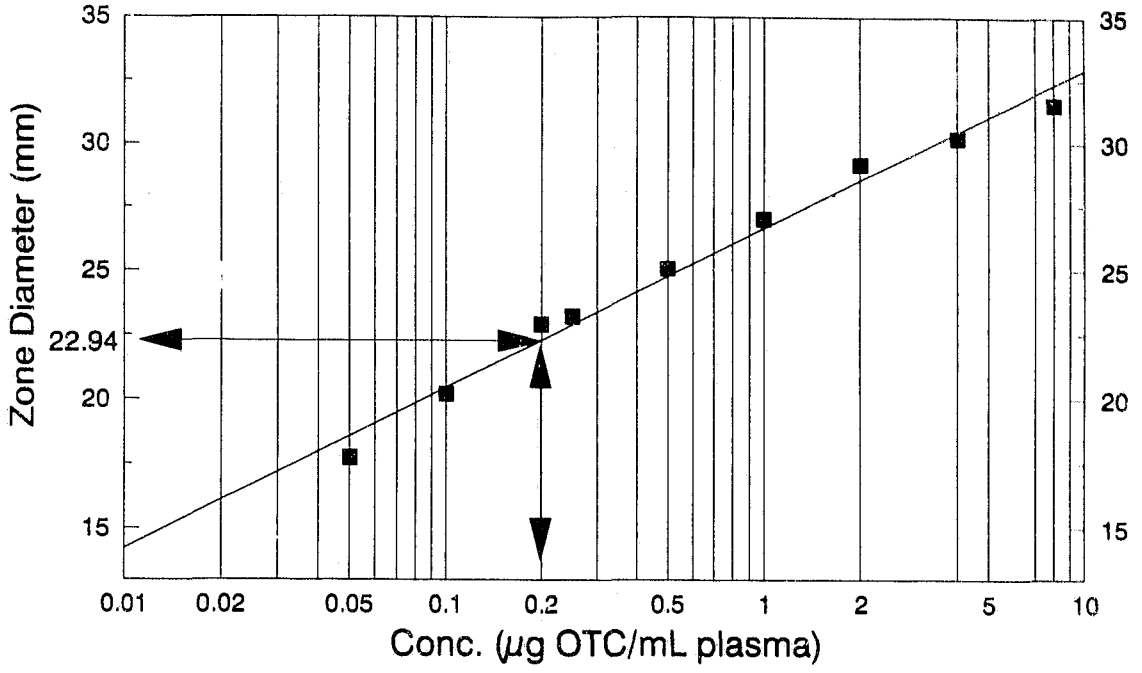

Figure 2. Standard response curve for oxytetracycline generated using a modified AOAC method. This curve was used to determine concentrations of oxytetracycline in plasma from Atlantic salmon during the pilot study.

calculated for each standard concentration. To determine the correction value, the average of the inhibition zone diameter of nine wells of the reference standard solution was subtracted from the zone diameter of the correction point for the standard response curve. (For example if the average for the 36 wells of 0.2 μg OTC/mL plasma reference standard was 20.0 mm and the average for 9 wells of 0.2 μg OTC/mL plasma reference standard was 19.8 mm, then the correction factor for the response curve was + 0.2 mm for the particular standard concentration on those three petri plates.) The correction value was then added to the zone diameter for that standard solution.

The corrected values for each standard solution, and the correction point for the reference standard solution 0.2 μg OTC/mL plasma, were plotted on semi-log paper, using logarithmic scale for oxytetracycline concentration and arithmetic scale for the average zone diameters. Using regression analysis a "line of best fit" was drawn. This was the standard response curve.

2.2.6 Determination of OTC Concentration in Fish Plasma: Assay of previously collected fish plasma samples was completed in the same manner as described for the standard response curve, including a negative control (plasma known to be oxytetracycline-free), as well as the reference standard solution. The reference point for the reference standard solution was determined from the standard response curve. If the unknown plasma sample had a larger average diameter zone of inhibition than

reference standard solution the difference was added to the diameter of the zone of inhibition of the **reference standard point on the response curve;** (Figure 2). (The reference point was 22.94 mm from response curve of 91/01/31). If the unknown plasma sample had a smaller average diameter zone of inhibition than the reference standard solution the difference was subtracted from the value of the diameter of the zone of inhibition of the **reference standard point on the response curve.** The concentrations of oxytetracycline corresponding to the corrected zone of inhibition diameter was read from the standard response curve.

2.3 Results and Discussion

Sensitivity of an assay is the lowest concentration of the sample that can be accurately measured, but this concentration may be several times higher than the limit of detection (71). Sensitivity should be at or below the lowest concentration of drug to be assayed. Throughout this study, the limit of detection was 0.01 - 0.05 μg OTC/ mL fish plasma, but the sensitivity of the assay was set at 0.10 μg OTC/ mL fish plasma, since the zones of inhibition for the 0.05 μG OTC/mL fish plasma exhibited a high degree of variability in successive assays. The equation for the regression line for the standard plasma curve was $y = 26.69 + 2.71x$ and the correlation coefficient was $r^2 = 0.986$ (Figure 2).

The standardization of the conditions of the assay were particularly important. Conditions considered were (a) constant incubation temperature (in stacks of petri dishes, the top and bottom usually heat fastest and the middle slowest); (b) inoculum (errors can result if the time elapsed between the first inoculated well and the last inoculated well exceeds 30 minutes); (c) beginning of incubation (bias caused by pre-diffusion of dishes prepared first); (d) uniform concentration gradients (thickness of agar). Any combination of these factors could influence the size of the zone of inhibition. To minimize the influence of the above in the assay, plates were inoculated in groups of 18, before establishing T = 0. Inoculation for each group of plates required less than 30 minutes. Following inoculation, the 18 plates were stacked in groups of three in the incubator.

The AOAC method presented a number of technical problems in the preparation of assay plates. First, agar thickness will influence zone diameter size. Thin layers give larger zones than thick layers. Nonuniformity in thickness will cause variability in zone size (49). Plates containing 20 mL of Difco Antibiotic Medium #8 were prepared 24 h in advance and held in an environmentally controlled room at +5.0° C until required. The previously prepared agar base plates were acclimated to room temperature for at least an hour before the *Bacillus cereus* seeded overlay was added to the surface of the plates. If the seeded overlay medium had been poured onto the surface of a cold agar base, the thin layer would have hardened too rapidly resulting in uneven assay plates. Secondly, there were problems in achieving

uniform sized wells. The agar wells tended to contract after the cork borer has been removed. This reduced the diameter of the wells by an average of 0.5 mm. In addition, if the pressure applied to remove an agar plug was too great, cracks would radiate from the well and the assay solution would tend to flow along these cracks resulting in variable zone diameters. Thirdly, when pipetting the 20 μL of agar into each well care had to be taken to avoid the formation of bubbles in the bottom of the well. Once the agar had hardened, any bubbles present in the well would break and this would allow the assay solution to flow along the bottom of the petri plate. Recovery of oxytetracycline, in such a sample, would be somewhat lower than expected.

Non-microbiological assays measure the total concentration of antimicrobial present in the sample, but in a microbiological assay it is the unbound antimicrobial which is measured. The tetracyclines exhibit a high degree of serum protein-binding. Protein-bound drug is not readily available for diffusion into the agar medium. As with biological systems, free drug is removed from the reservoir, protein-bound drug will then be released from serum proteins, and become available for diffusion throughout the system (9, 10, 72). While this will extend the period of time in which drug will be present within the biological system, it will not be reflected in the zone of inhibition for a specific point in time in a microbiological assay. The zone of inhibition will be a reflection of the "free", unbound drug (9, 20). Oxytetracycline is known to bind to tissue and plasma proteins in fish (11). However, since the

unknown concentrations of drug in fish plasma were determined from a standard response curve generated from known concentrations of oxytetracycline in fish plasma, the degree of protein-binding should have been similar. The zones of inhibition would be indicative of the total available concentration of drug at the point in time at which the sample was collected.

Specificity has been reported to be low for microbiological assays (11, 24, 65). The assays are susceptible to interference by microbiologically active break-down products or metabolites. Researchers using HPLC techniques have reported that no detectable metabolites of oxytetracycline were found in the fluids or tissues of either Atlantic salmon or rainbow trout (26, 74). This would suggest that the modified microbiological assay used in the present experiments would determine a drug concentration that would reflect only oxytetracycline present in the plasma. Even with the inclusion of metabolites in the total concentration of antibiotic, Bruno (18) felt that microbiological assays were superior to HPLC since only the biologically active component(s) rather than the total assay solution concentration is measured.

The Association of Official Analytical Chemists microbiological assay for oxytetracycline in feeds was successfully modified to convert the assay to a well-diffusion assay and to use the modified assay for the determination of oxytetracycline in fish plasma.

3.0 PHARMACOKINETICS OF OXYTETRACYCLINE IN PLASMA OF ATLANTIC SALMON: A PILOT STUDY

3.1 Introduction

Pharmacokinetic research in teleosts is limited. In fish, the pharmacokinetics of an antimicrobial is dependent on drug dose (48, 69), gastric evacuation rate (17, 29), and the absorption, distribution, metabolism and excretion rate of the drug. All of these parameters are temperature dependent (12).

The ubiquitous contact with all body tissues and ease of collection encourages the use of blood components (ie, plasma), as indicators of the functional state of vertebrate biological systems. The validity of this practice is based on several assumptions; that neither the sampling procedure nor the subsequent sample treatment will significantly alter the *in vivo* characteristics of the variable of interest. With fish, however, anaesthesia (40), method of euthanasia (67, 68), fish handling (79), stress or exposure to the air (38, 70) can result in haemoconcentration, electrolyte shifts, hyperglycaemia, hypercholesterolemia, or acidosis. Sampling (handling) procedures, type of anticoagulant used, sample storage time and temperature (52), and type of storage container (2), may also influence sample quality.

A study was undertaken to determine the effect of storage conditions of plasma samples on oxytetracycline concentration. A pilot study was conducted to evaluate the suitability of sampling times, and trends in the kinetics of oxytetracycline in plasma following a single intraperitoneal (IP) or a single oral (PO) administration of antibiotic at a high (200 mg OTC/ kg body weight), and a low (100 mg OTC/ kg body weight) dose rate.

3.2 Material and Methods

3.2.1 Experimental Evaluation of Storage Containers for Oxytetracycline: Blood samples were collected and handled as described in section 3.2.3, frozen at - 20° C and stored at - 70° C pending analysis. Atlantic salmon plasma was spiked with oxytetracycline hydrochloride (Pfizer Ltd) dissolved in 0.1N HCL to final plasma concentrations of 0.05, 0.2, 1.0 and 8.0 μg OTC/mL. Aliquots of each spiked plasma (0.2 mL for each of 0.05, 1.0 and 8.0 μg OTC/mL solution and 1.0 mL for the 0.2 μg OTC/mL solution) were transferred into previously labelled sterile 10 mL glass vials and 2 mL polypropylene vials. Eight glass vials and eight polypropylene vials per concentration were stored at - 70° C. Using the modified AOAC microbiological assay described in Chapter 2, the concentration of oxytetracycline in the plasma was determined on a weekly basis for a period of seven weeks.

3.2.2 Experimental Fish: Fifty, first sea year Atlantic salmon weighing an average of 468.1 ± 112.7 g (mean ± 1SD) were obtained from a Bay of Fundy seacage site (Sea Farms Canada Inc., Saint John, New Brunswick). The fish were maintained in 1.5 m diameter fibreglass tanks in recirculating, reconstituted seawater (Instant Ocean, Aquarium Systems, Mentor, Ohio), salinity 24-29 ppt, at 10° C, under seasonal light conditions. Fish were fed to satiation with 5 mm Fundy Choice pelleted dry feed (Corey Feed Mills Ltd, Fredericton, N.B.). After a three week period of acclimation, fish were anaesthetized with MS 222 (Syndel Laboratory Ltd., Vancouver, BC) at a concentration of 100 mg/L, weighed and individually identified via cold-branding (Appendix A). Fish were acclimated for an additional two weeks prior to treatment.

3.2.3 Experimental Procedure: Prior to dosing fish were starved for two days and then placed into aerated holding tanks. Individuals receiving per os treatments were dip-netted, manually restrained by being wrapped securely in netting and then placed into a foam lined V-shaped container. Identified by their freeze-brand, each fish received a dose of oxytetracycline hydrochloride (Pfizer Ltd.) based on the individual's previously recorded weight. Ten fish received oxytetracycline in a gelatin capsule at a dose rate of 200 mg OTC/kg body weight via gastric intubation. In order to facilitate detection of regurgitation, red food colouring (Barbours Ltd., Sussex, N.B.) was added to the capsule contents. Ten additional fish received a slurry of oxytetracycline hydrochloride suspended in 0.2% (w/v) aqueous Bacto-Agar at a dose

rate of 100 mg OTC/kg of body weight via intra-oesophageal intubation. Oxytetracycline hydrochloride dissolved in filter sterilized phosphate buffered saline (D-PBS) (pH 7.5) was administered via intraperitoneal injection to two groups of ten fish. Fish to receive intraperitoneal injections were securely wrapped in netting, presented ventral surface up and injected on the midline near the pelvic fins. Ten fish received an injection of oxytetracycline at a dose rate of 200 mg OTC/kg body weight and a second group of ten fish received an injection at a dose rate of 100 mg OTC/kg body weight. Ten control fish received an injection of an equivalent volume of sterile D-PBS.

Fish were euthanized by a sharp blow to the dorsal surface of the head. Blood samples were collected via cardiac puncture (through the mouth) and/or by puncture of the caudal vertebral sinus using sterile (lithium heparin) vacutainors (Becton-Dickinson, Rutherford, New Jersey) equipped with 20 gauge (25 mm) needles (Becton-Dickinson, Rutherford, New Jersey). Blood samples were collected at 1, 3, 6, 12 and 36 hours after administration. Samples were kept on ice prior to centrifugation (7500 RCF) for 10 minutes at room temperature (18° C - 20° C) in a Beckman TJ-6 centrifuge. Plasma was transferred into labelled sterile 16 x 125 mm polypropylene tubes using sterile pipettes. Plasma samples were frozen at - 20° C and stored at - 70° C pending analysis.

3.2.4 Analysis of Plasma Oxytetracycline: Plasma oxytetracycline was assayed as described in Chapter 2.

3.2.5 Statistical Analysis: Statistical analysis was performed using Minitab Statistical Package, Release 7.1 (62). The statistical analysis of storage conditions, the effect of OTC concentration, storage container material and duration of storage on the diameter of the zone of inhibition (OTC concentration) was by multiple polynomial regression. Interactions between the independent variables (ie, concentration, material, duration) were assessed using partial F-tests (31, 50). The effect of dose and route of administration on the concentration of OTC in plasma was compared using analysis of variance (62). Results of the analysis were deemed significant if $p \leq 0.05$.

3.3 Results

3.3.1 Storage Conditions: The influence of duration of storage on concentration of oxytetracycline in fish plasma stored in polypropylene or in glass vials is presented in Figures 3 and 4 respectively. Polynomial regression analysis was performed on all data. When a Fischer exact test (80) was performed, there was no significant difference ($p \leq 0.05$) between the slopes of regression lines. No statistically significant reduction in the concentration of oxytetracycline in fish plasma, in either polypropylene ($p = 0.630$) or glass ($p = 0.696$) containers, occurred over the seven

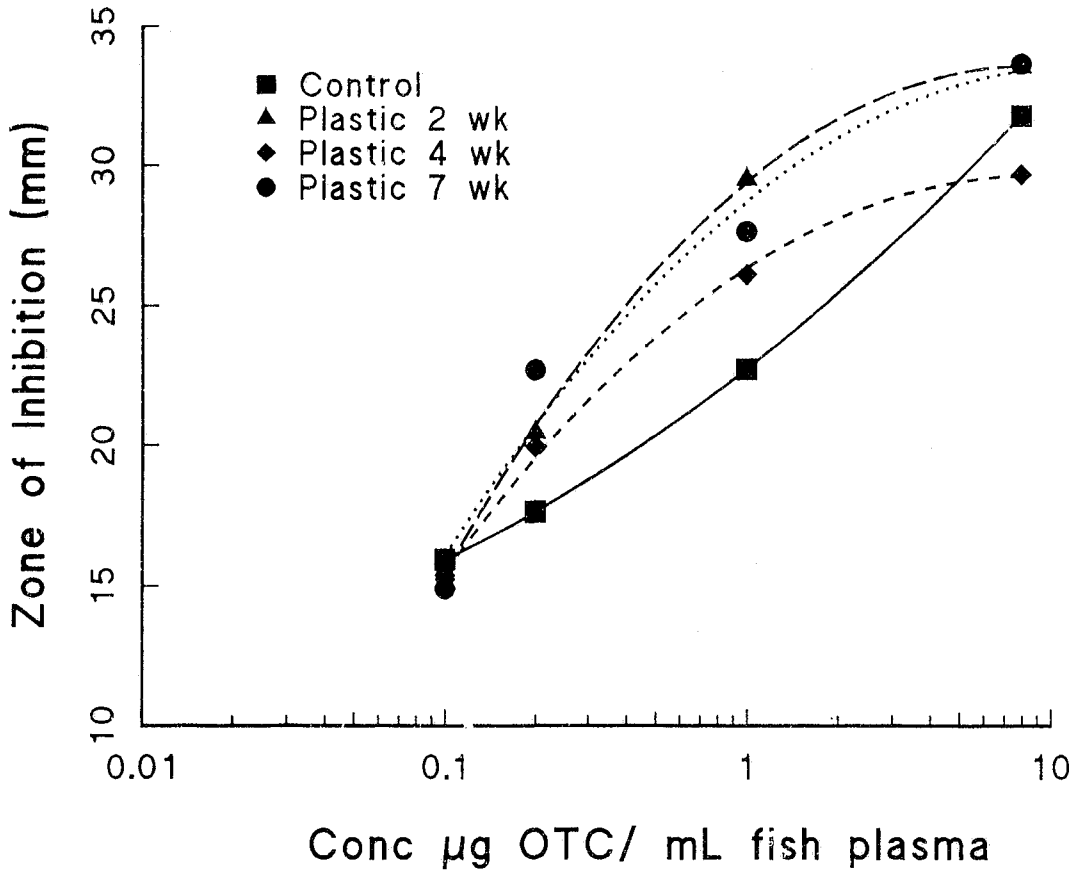

Figure 3. Concentration of oxytetracycline in plasma, stored in polypropylene vials over seven weeks.

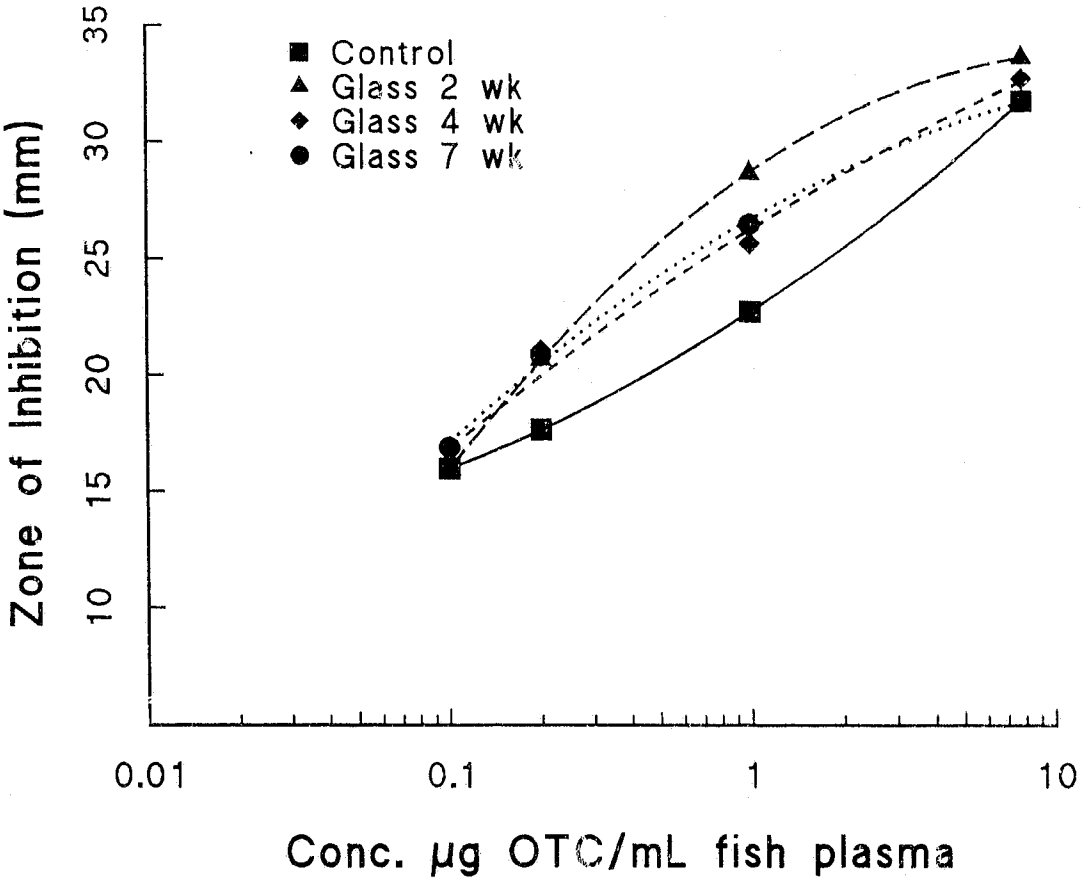

Figure 4. Concentration of oxytetracycline in plasma, stored in glass vials for seven weeks.

weeks of the trial. No significant difference was demonstrated between the use of glass or polypropylene containers ($p = 0.893$) as storage containers for oxytetracycline in fish plasma at - 70° C for seven weeks.

Polypropylene or polyethylene vials and labware for preparation and storage of solutions containing low concentrations of antibiotic were recommended (2), due to possible adsorption of the antimicrobial to the walls of glass containers. Previous researchers reported that there was no observable degradation of oxytetracycline in plasma samples stored at - 20° C after ten weeks (47), or in rainbow trout muscle stored at - 20° C after 21 - 23 weeks (39). The type of storage container used in these studies was not reported.

3.3.2 Pharmacokinetics: Pilot Study: Plasma OTC concentrations as a function of time are presented in Figure 5. Data are from the three groups of fish that had received the agar slurry (100 mg OTC/kg) PO, a high dose of oxytetracycline IP and a low dose of oxytetracycline IP. An average plasma concentration of 0.61 ± 0.46 μg OTC/mL (mean ± 1SD) was measured in fish which had received an intraperitoneal administration of oxytetracycline at a dose rate of 200 mg OTC/kg body weight. Fish which had received an intraperitoneal administration of oxytetracycline at a dose rate of 100 mg OTC/kg body weight had an average plasma concentration of 0.25 ± 0.35 μg OTC/mL (mean ± 1SD). Fish which received an oral administration of oxytetracycline in the aqueous Bacto-agar slurry at a dose rate

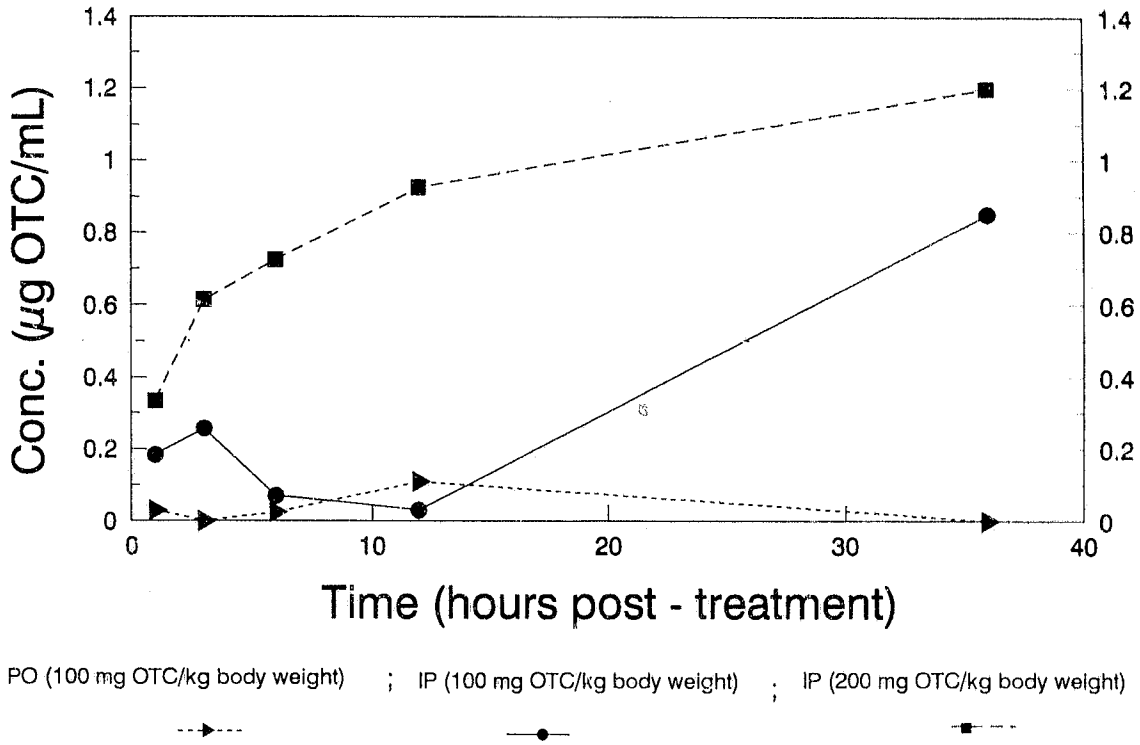

Figure 5. Plasma concentration of oxytetracycline in Atlantic salmon following a single administration via intraperitoneal injection (IP) or by oral gavage (PO).

of 100 mg OTC/kg body weight, had an average plasma concentration of 0.02 ± 0.04 μg OTC/mL (mean ± 1SD) for 36 h. There was no significant interaction between the initial concentration of oxytetracycline and the route of administration for the intraperitoneal injection ($p = 0.066$). No comparison of the effect of dose rate for the oral route of administration was possible since there were no detectable levels of OTC in the plasma of fish receiving the gelatin capsules, dose rate 200 mg OTC/kg body weight. Route of administration at a dose rate of 100 mg OTC/kg body weight did have a significant influence ($p < 0.001$) on the mean concentration of oxytetracycline achieved in the plasma of Atlantic salmon at 10° C.

There were difficulties in collecting adequate volumes of blood from fish that were < 0.4 kg body weight, had not eaten for an extended period of time, or had been freeze-branded for two seconds. The latter fish had probably been stressed as a result of extended confinement in holding tubs prior to dosing (81). Generally, less than 3 mL of blood was collected from each individual fish sampled within the first three hours post-treatment while sample volumes collected after three hours post-treatment were 6 - 11 mL. Since only a limited volume of blood was collected at time 0 and the 3 h sample collection times, dual site sampling, via both cardiac puncture and venipuncture of the ventral sinus of the caudal peduncle, was instituted beginning with the 6 h sampling time.

Oxytetracycline was detected in the plasma from two of the control fish. Control fish and fish injected with the high dose were part of the group which had been branded for only two seconds. The two second brands had faded making it difficult to accurately identify the fish. As a consequence, two control fish may well have received injections intended for the high dose group of fish. Fish from the high dose injection group did not have any detectable oxytetracycline in the plasma samples, demonstrating that incorrect reading of faded cold-brands generated some confusion between the two groups of fish.

3.4 Discussion

Plasma concentrations of chemotherapeutics are maximized by a direct administration of drug into the circulatory system, but for fish this method tends to be too stressful since it requires extensive handling as well as removal from water. Oral administration of oxytetracycline, in the feed, is the most common delivery system. Specific variables which have been shown to affect the absorption and clearance of xenobiotics ingested by fish include: composition of the diet (86); characteristics of the feeding pattern such as meal frequency and daily ration (30); variations in the level of digestive enzymes (61); characteristics of the gut environment, such as the presence of various cations, which may affect ability of the drug to be absorbed (57); the post-absorptive mechanisms that influence the amount and form of chemical that is transported to tissues (ie, hepatic clearance, metabolism

and bilary excretion) (68); duration of exposure; environmental concentrations and temperature (22).

Oxytetracycline administered to Atlantic salmon in seawater at 10° C via intraperitoneal injection (Figure 5) appears to enter the blood compartment at a slower rate than expected. A peak serum concentration of OTC, following an intraperitoneal administration at a dose rate of 20 mg OTC/kg body weight, was reported for Atlantic salmon held in freshwater at 7.0° C of 1.4 μg OTC/mL (18). This was very similar to the apparent peak concentration of 1.2 μg OTC/mL plasma at 36 h for the high IP dose group of Atlantic salmon. Plasma concentration in the low dose IP group was apparently still increasing at 36 h. Further research would be required, utilizing sampling times which extend past the 36 h limit set in this study, in order to confirm when the peak concentration of oxytetracycline would occur.

The length of time that foodstuffs, or medicated feed, are contained within the gastro-intestinal tract will affect the degree of absorption that can occur. In poikilothermic species, gastric emptying rate (GER) is influenced by environmental temperature, food type and presentation, fish size, method of feeding and feeding history of the fish (17, 29, 30, 85, 86). There is an initial lag period based on the digestibility of the feed and to allow time for the feed pellets to be moistened in the stomach. For fully acclimated fish, excretion is an almost continuous process with a reported GER of 0.2 - 0.7 g of feed/h for rainbow trout at 10° C (30). Mean transit

time for the gastro-intestinal tract (10° C - 11° C) has been reported as 27.7 - 34.0 h (28) and 46 h (28) in rainbow trout and 37.8 h in sockeye salmon (17). More recent research by Usher *et al.* (83), suggests that a salinity-dependent increase in gut evacuation rate exists. They postulate that gastric evacuation rate is greater in seawater adapted versus freshwater adapted Atlantic salmon smolts. The thirty - six hour sampling time frame selected for this experiment should have been appropriate for both the slurry and the gelatin capsule administration.

The administration of an exact dosage of chemotherapeutic is effectively guaranteed by utilizing gelatin capsules. There does not appear to be anything in the composition of capsules (gelatin, FD&C colouring agent, an opacifying agent, 0.15% sulphur dioxide to prevent decomposition during manufacture, and 12-16% water, depending on storage conditions) which would suggest slow dissolution in the stomach of fish. But, throughout this experiment it was close to 36 hours before any oxytetracycline was detected in the plasma of fish which had received oxytetracycline in gelatin capsules. It has been reported that force-feeding frequently decreases the rate of evacuation (29) and this may partially explain the apparent delay in dissolution of the gelatin capsules. Previous research administering oxytetracycline in gelatin capsules to rainbow trout (8° C) exhibited a peak concentration on the third day post administration (47), while whole-body autoradiography of fish held at 6° C (47) found that there had been negligible absorption of oxytetracycline at 48 h and it was not until day seven post administration that radio-labelled oxytetracycline

exhibited wide distribution. The short time frame used in this study was inadequate for the evaluation of oxytetracycline administered via gelatin capsule.

Enzymatic hydrolysis is required before capsule dissolution can occur. At present, it is not known whether pepsins I (a salt-activated pepsin) and II are present in the stomach of Atlantic salmon, but Sanchez-Chiang *et al.* (75), described the presence of both pepsins in the stomach of seawater-adapted adult chum salmon, *Oncorhynchus keta*. There are no significant differences in the activities of digestive enzymes, nor in the pH optima for the activity of proteolytic enzymes in the gut in feeding seawater- or freshwater- adapted Atlantic salmon smolts (83). Several of the fish in the capsule treatment group had not been on feed for approximately 2 - 3 weeks. Long periods of food deprivation may induce general changes in hepatobiliary function (21, 29). Digestive enzymes would be present in the stomach, even under anorexic conditions, but may have been reduced, causing a delay in the ability to dissolve the gelatin capsules (83). The OTC-slurry preparation (13) for oral administration was the more effective delivery system and recommended for future experimentation.

Removal of fish from the water induces the release of elevated levels of catecholamines ($> 10^{-6}$ M) particularly adrenaline within minutes (32). The effect may last from hours to three days in teleosts before catecholamine levels return to normal resting levels (58, 81). While there is some controversy surrounding the

action of catecholamines in teleosts, the net effect appears to be increased aortic pressure, increased heart stroke volume and constriction of blood vessels and osmoregulatory disruptions (58). It may be that vasoconstriction of blood vessels was responsible for some of the problems associated with blood sample collection.

In summary, this study has shown: 1) that storing of plasma samples in polypropylene vials at - 70° C for up to seven weeks does not result in degradation or loss of the antibiotic and 2) that of the two methods examined, an agar gel slurry of oxytetracycline is the better carrier for oral administration to Atlantic salmon held in seawater. Since the sampling times selected were not sufficient, it was impossible to determine pharmacokinetic trends beyond an indication that there was low apparent bioavailability for oxytetracycline in Atlantic salmon held in seawater.

4.0 PHARMACOKINETICS AND BIOAVAILABILITY OF OXYTETRACYCLINE IN ATLANTIC SALMON HELD IN SEAWATER AT DIFFERENT TEMPERATURES

4.1 Introduction

Modern salmonid aquaculture farms rely on high stocking densities in order to be competitive. A disease outbreak under these circumstances can be economically disastrous. To counteract disease episodes and reduce losses, aquaculturists rely on the use of chemotherapeutics, particularly antibiotics. Dosage regimes used in aquatic systems frequently rely on information generated for mammalian systems and this may not be appropriate. The use of antibiotics also introduces public health considerations, specifically residue concentrations in the tissue of marketed fish. Information on the pharmacokinetics and bioavailability of antibiotics in finfish under different environmental conditions is limited. To minimize potential human and aquatic impacts, and maximize efficacy, detailed pharmacokinetic investigations of chemotherapeutics in finfish are needed.

Oxytetracycline, one of the most commonly used drugs in aquaculture, is a broad-spectrum antimicrobial with considerable activity against Gram-negative

bacteria. Other researchers have investigated the elimination pharmacokinetics of oxytetracycline in fish after intravascular and oral administration (12, 33, 35, 69), but all of these studies were conducted in freshwater systems. Atlantic salmon are usually cultured in seawater. To date, there has been no study of plasma elimination kinetics or bioavailability of oxytetracycline in Atlantic salmon under seawater conditions.

The objective of the present study was to compare single intravascular administration of oxytetracycline and a single oral administration of drug to determine the plasma pharmacokinetics and bioavailability of oxytetracycline in adult Atlantic salmon held in seawater at 10° C and 15° C. An intra-arterial administration was selected to establish baseline pharmacokinetic parameters, while the oral route of administration was evaluated because the current conventional delivery system for antibiotics to aquatic species is in medicated feed. Indwelling aortic cannulas, which facilitate blood collection from individual animals with minimal stress from handling, were used for intravascular administration of drug and for serial blood sampling. A temperature of 15° C was chosen because it approximated upper temperature levels which would be experienced by Atlantic salmon held in seacages (Bay of Fundy), while 10° C represented the mid-range temperature of the Atlantic salmon in aquaculture (76). Disease outbreaks, for which an administration of oxytetracycline would be given, most commonly occur between these two temperatures.

4.2 Materials and Methods

4.2.1 Experimental Fish: Atlantic salmon weighing 470.1 ± 39.9 g (mean ± 1SD) were obtained from the Salmon Development and Demonstration Farm, St. George, New Brunswick. The fish were acclimated to recirculating seawater reconstituted using Instant Ocean (Aquarium Systems, Mentor, Ohio), salinity 24.0 - 29.0 ppt at 10° C and 15° C, in 2 m diameter fibreglass tanks, under an incandescent light source of low intensity with a photoperiod of 12 h light and 12 h dark, for a minimum period of three weeks prior to surgery. Fish were fed once daily to satiation with 5 mm Fundy Choice pelleted dry feed (Corey Feed Mills Ltd., Fredericton, N.B.).

4.2.2 Experimental Design: Atlantic salmon were randomly assigned to temperature and treatment groups using a list of previously generated random numbers (Minitab Statistical Package, Release 7.1). One group of sixteen fish was maintained at 10° C, while the second group of sixteen fish was maintained at 15° C. Weights of individual fish were recorded and cannulas implanted in the dorsal aorta (see section 4.2.3). Immediately prior to full recovery from anaesthesia, fish were treated with either the vehicle or oxytetracycline. One group of five fish received an injectable veterinary formulation Oxyvet 100^{R} (Sanofi Sante Animale, Canada Ltd.) at a dosage of 100 mg OTC/ kg body weight, intra-arterially through the cannula. The corresponding three control fish received an equivalent volume of sterile heparinized saline (280 IU heparin/mL in 0.85% saline). Five fish were medicated with a slurry of

oxytetracycline hydrochloride (Pfizer Ltd.) suspended in 0.2% (w/v) aqueous Bacto-Agar (100 mg OTC/kg of body weight) via gastric intubation. The volume of the medicated slurry administered to each fish was based on the recorded individual weight. Three control fish received equivalent administrations of seawater via gastric intubation.

4.2.3 Surgical Procedure: A 74 cm cannula (PE50, Clay Adams Intramedic™, ID 0.58 mm, OD 0.97 mm) was assembled during surgery from a 14 cm proximal section and a 60 cm distal section. Prior to surgery, suture material (2.0 Prolene, Ethicon Inc., Sommerville N.J.) was securely tied around a 1 cm reinforcement collar of PE 190 tubing glued (Nexaban™, cyanoacrylic glue, RX Medical Inc., Raleigh, N.C.) at the mid-point of the proximal section of the cannula. During surgery, the proximal and distal sections were connected internally with a 2 cm section of 23 gauge stainless steel needle. This internal connection was protected with an external 3 cm collar of PE 190 tubing attached with cyanoacrylic glue (Krazy Glue®, The Borden Co. Ltd., Japan). The distal end of the cannula was attached to a three-way valve in a styrofoam float (Figure 6).

Prior to and during surgery, fish were anaesthetized with MS - 222 (tricaine methane sulfonate, Syndel Laboratory Ltd., Vancouver, B.C.) at 100 and 50 mg/l, respectively. An internal trocar (D'Addario PL 020 guitar string) was inserted into the 14 cm proximal section of the cannula. The end of the cannula was positioned

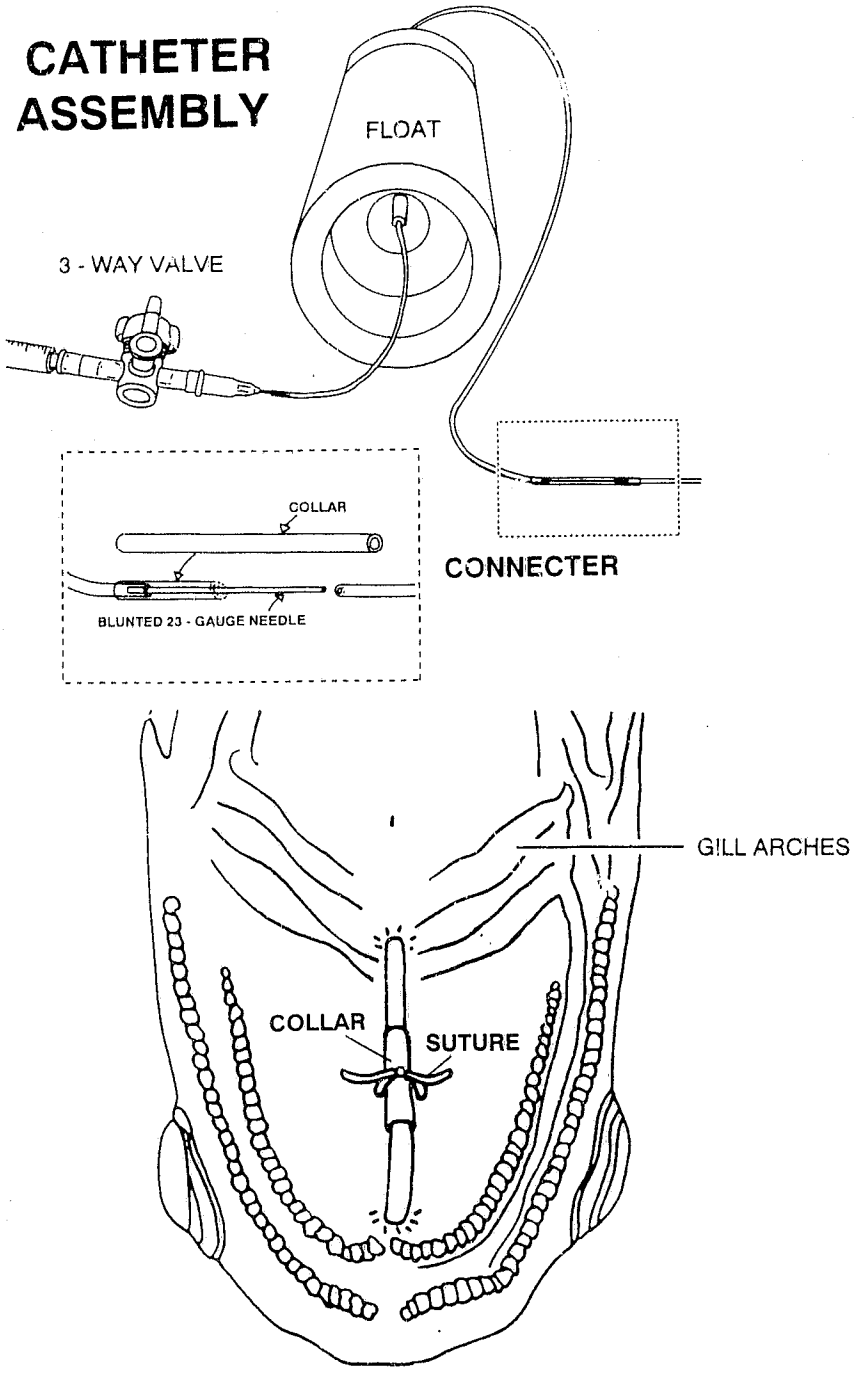

Figure 6. Catheter assembly used in the cannulation of Atlantic salmon

on the midline of the palate between the second and third gill arches and gently pushed through the palate at a 45° angle (60). The cannula tip was advanced 6 cm (approximately 20% of the fork length of a 500 g fish) into the dorsal aorta. The internal trocar was removed and the distal end of the 14 cm segment of the cannula was passed through a 14 gauge needle which had been previously inserted through the cartilage of the snout. The proximal and distal sections of the cannula were connected internally and the protective collar was glued in place (Figure 6). Patency was confirmed by withdrawal of blood. The cannula was flushed with 0.5 mL of sterile heparinized saline (280 IU heparin/mL of 0.85% saline). The cannula was sutured to the hard palate with the suture material attached prior to surgery. The cannula was further stabilized by the placement of two additional suture supports, one subcutaneously immediately caudal from the head and the second through the superficial dorsal musculature cranial to the dorsal fin. The suture supports consisted of 4-0 Superamid White (Serag - Wiessner Nail) previously inserted inside a 6-7 cm length of PE 190 tubing. The suture support was securely attached at approximately the middle of the protective collar of the cannula. Upon recovery each fish was housed in one quarter of a 2 m diameter fibreglass tank which had been partitioned into quarters with 2.5 cm mesh Vexar™. One or more non-cannulated fish were housed with each of the surgical fish, because it had been previously noted that cannulated fish appeared to recover more quickly when companion fish were present. The same procedure was followed for all fish.

4.2.4 Experimental Procedure: Blood samples (0.5 mL) were collected from each fish at 0, 3, 6, 12, 24, 36, 48, 96 and 192 hours after treatment using sterile 1 mL syringes, which had been pre-flushed with sterile heparinized saline solution (140 IU heparin/mL of 0.85% saline), attached to a three-way valve. The first 0.5 mL of blood plus the heparinized saline solution in the cannula was held in the first syringe, while the blood sample was collected using a second syringe. Following withdrawal of the blood sample, the initial 0.5 mL of blood plus heparinized saline solution was gently flushed back into the dorsal aorta, followed by 0.4-0.8 mL of freshly prepared sterile heparinized saline solution (140 IU heparin/mL of 0.85% saline). Samples were transferred into sterile serum (no-additive) vacutainors (Becton-Dickinson, Rutherford, New Jersey) and kept on ice until centrifuged for 10 minutes at 7500 RCF in a Beckman TJ - 6 centrifuge. Plasma was transferred to labelled sterile 16 x 125 mm polypropylene tubes using sterile Pasteur pipettes. Plasma samples were initially frozen at - 20° C and, within 24 h, stored at - 70° C pending analysis.

The plasma oxytetracycline concentration was determined as previously described (see Chapter 2). All plasma samples were assayed within one month of collection.

4.2.5 Pharmacokinetic Analysis: The pharmacokinetics of plasma oxytetracycline were determined using the computer software package (PKCALC^{R}) (R.C. Shumaker, Merrell Dow Pharmaceuticals Inc, 1986) (77). The equations used in the software

are listed in Appendix B. Calculated pharmacokinetic parameters include area under the concentration versus time curve (AUC), time to maximum plasma concentration (T_{max}), maximum plasma concentration (C_{max}), plasma clearance (Cl), terminal half - life of elimination ($T_{1/2}$), and volume of distribution at steady state (VD_{SS}). A best fit compartmental analysis was performed on mean data from each treatment group. Bioavailability (BA) for both 0-24 h (15° C) and 0-192 h (10° C and 15° C) were calculated using the formula: $BA\% = (AUC_{PO} / AUC_{IA}) \times 100$.

4.2.6 Statistical Analysis: The effects of route of administration and temperature on individual pharmacokinetic parameters, except bioavailability, were determined using factorial analysis (31) and a general linear model (62). The effect of route of administration and temperature on bioavailability were analyzed, using a regression model coupled with a logarithmic transformation to stabilize the variance. Confidence intervals (95%) were calculated for the 0-192 h bioavailability data (10° C and 15° C) and the 0-24 h bioavailability data (15° C). Results of analyses were deemed significant if $p \leq 0.05$.

4.3 Results

4.3.1 Pharmacokinetic Parameters: Mean plasma concentrations following intra-arterial and oral administrations of oxytetracycline at 10° C and 15° C are presented in Figure 7. Pharmacokinetic parameters (0-192 h), including area under the concentration-time curve, maximum plasma concentration, time to maximum plasma

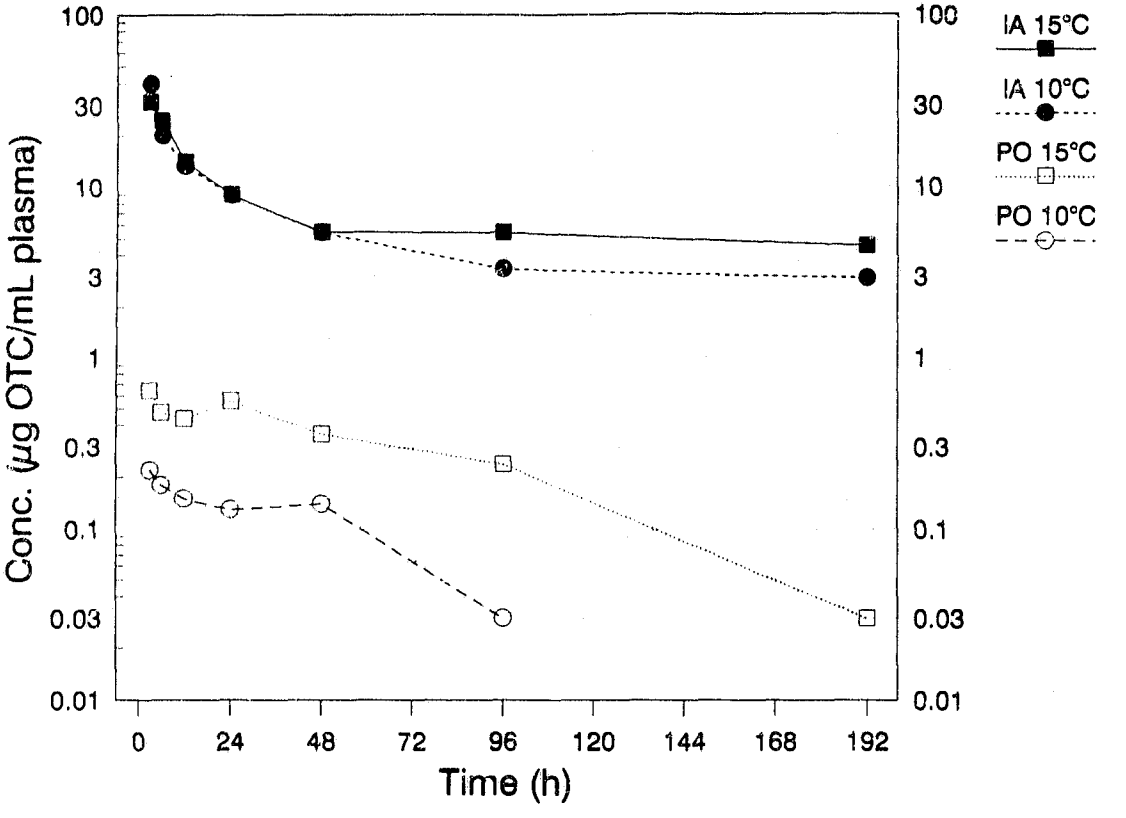

Figure 7. Plasma concentration of oxytetracycline (dose = 100 mg OTC/kg body weight) following a single bolus administration intra-arterial (IA) or oral (PO) to Atlantic salmon held in seawater at 10° C and 15° C.

concentration, apparent volume of distribution at steady state, half-life of elimination, plasma clearance and oral bioavailability for oxytetracycline are presented in Table I. Values for C_{max} and T_{max} were read directly from the concentration time curves. Since the first sample was not collected until three hours post treatment these values were considered estimates for both parameters.

The apparent oral bioavailability of oxytetracycline (t = 0-192 h) for Atlantic salmon held at 15° C was 7.4% (95% confidence intervals {4.1%, 13.6%}), and for 10° C was 1.5% (95% confidence intervals {0.8%, 2.7%}) (Table I). A general linear model (GLM) was used to test for significance between IA and PO routes of administration as well as for differences between the 10° C and 15° C data. In the statistical evaluation of the regression equation, one observation from the 10° C oral data yielded a Cook's D statistic[1] of 1.1 (31, 50), suggesting that it was an outlier with a high influence on the estimate of the bioavailability ratios. This is because a large mean for the AUC_{IA} denominator relative to the small mean AUC_{PO} numerator meant that a small error in the denominator could distort the analysis. Including the outlier in the model (coefficient of determination {predicted Log AUC vs residuals for observed log AUC} R^2 = 75.9%) showed that only the route of administration of oxytetracycline was significant (Table II). Including the outlier indicated that

[1]Cook's D Statistic measures how much the regression coefficients are changed by deleting one observation from the data set. The observation may be deleted because it differs greatly from the other observations or because it's deletion changes the predicator coefficient greatly. Both factors may be present. If the model is correct the Cook's D statistic should be less than 1.0.

Table I. Pharmacokinetic parameters for oxytetracycline following intra-arterial and oral administration (100 mg OTC/kg body weight) to Atlantic salmon (mean ± 1SD; 0 - 192 h)

Temperature	10 ° C	10 ° C	15 ° C	15 ° C
Route	IA	PO	IA	PO
Av. Wt.	486.5 ±14.3	478.6 ±47.4	481.8 ±40.0	475.4 ±33.6
Number Fish	4	2	4	3
AUC	1247.4 ±89.4	18.8 ±5.1	1366.5 ±570.3	97.8 ±30.8
C_{max}	39.9 ±11.1	0.22 ±0.0	31.5 ±6.1	0.77 ±0.4
T_{max}	3.0 ±0.0	3.0 ±0.0	3.0 ±0.0	3.0 ±0.0
VD_{SS}	7.5 ±2.04	-1.701412E+38	5.95 ±1.93	-1.701412E+38
$T_{1/2}$	62.2 ±14.2	72.5 ±8.4	58.3 ±35.5	171.6 ±89.6
Cl	80.5 ±5.96	5522.0 ±1509.1	85.8 ±41.7	933.4 ±143.2
BA		1.5 (0.8, 2.7)		7.4 (4.1, 13.6)

IA		Intra-arterial route of administration
PO		Oral route of administration
Av. Wt.	(g)	Average Weight
AUC	(µg-h/mL)	Area under the concentration time curve
C_{max}	(µg/mL)	Maximum plasma concentration
T_{max}	(h)	Time to maximum plasma concentration
VD_{SS}	(L/kg)	Apparent volume of distribution at steady state
$T_{1/2}$	(h)	Elimination half - life
Cl	(mL/h)	Plasma clearance
BA	(%)	Oral Bioavailability

Table II. Significant predictors ($p < 0.05$) for pharmacokinetic parameters (0-192 h) of oxytetracycline following intra-arterial and oral administration. Analysis performed using General Linear Model (includes outlier*).

	Means ± 1SD					
	Route			Temperature		
Parameter	IA	PO	p	10 ° C	15 ° C	p
AUC	1306.9 ±119.2	150.7 ±137.6	0.000	717.4 ±128.1	740.2 ±128.1	0.902
C_{max}	35.7 ±2.5	0.5 ±2.9	0.000	20.4 ±2.7	15.8 ±2.7	0.247
T_{max}	6.5 ±2.5	3.0 ±2.5	0.155	6.5 ±2.5	3.0 ±2.5	0.508
VD_{SS}	6.7 ±0.5	-1.7 ±0.6	0.000	2.9 ±0.6	2.1 ±0.6	0.317
$T_{1/2}$	60.2 ±157.5	-151.8 ±181.9	0.397	-183.2 ±169.3	91.7 ±169.3	0.273
Cl	83.2 ±7.8	6.5 ±9.0	0.000	42.1 ±8.4	47.6 ±8.4	0.653
BA			< 0.001			0.630

* Outlier in 10° C oral route of administration data previously identified using a Cook's D statistic (see section 4.3.2)

the route of administration had a significant influence for all parameters except $T_{1/2}$ and T_{max}, while temperature had no impact on the plasma pharmacokinetics of oxytetracycline in Atlantic salmon held in seawater (Table II). Analysis showed that when the previously identified outlier was removed, route of administration was significant for all pharmacokinetic parameters, while temperature had a significant ($p \leq 0.05$) influence only on bioavailability and T_{max} (see Table III). The data used for the analysis throughout the remainder of this study excluded the outlier. Further analysis of the data using a factorial analysis indicated that there were simple temperature effects on all pharmacokinetic parameters except for AUC and plasma clearance.

4.3.2 Determination of Compartmental Model: Best fit compartmental analysis was performed on each set of data as well as on the mean data for both 10° C and 15° C following an intra-arterial administration of oxytetracycline (Table IV). Data were fitted to several compartmental models. Differences between observed and predicted concentrations of oxytetracycline were expressed as percent deviation. The percent deviation (Tables IV and V) and coefficient of determination (r^2) of the observed data to the model predicted values (Tables VI) were used to determine the model which best described the pharmacokinetics of oxytetracycline in Atlantic salmon.

Table III. Significant predictors ($p < 0.05$) for pharmacokinetic parameters (0-192 h) of oxytetracycline following intra-arterial and oral administration. Analysis performed using General Linear Model (outlier* removed).

	Means ± 1SD					
	Route			Temperature		
Parameter	IA	PO	p	10 ° C	15 ° C	p
AUC	1306.9 ±113.5	55.8 ±142.7	0.000	629.3 ±132.8	733.4 ±120.4	0.570
C_{max}	35.7 ±2.6	1.02 ±3.3	0.000	20.9 ±3.1	15.8 ±2.8	0.245
T_{max}	3.0 ±0.0	3.0 ±0.0	0.000	3.0 ±0.0	3.0 ±0.0	0.000
VD_{SS}	6.7 ±0.7	-1.8 ±0.7	0.000	3.0 ±0.7	2.1 ±0.6	0.327
$T_{1/2}$	60.2 ±18.9	128.5 ±24.1	0.045	77.0 ±22.4	111.7 ±20.3	0.271
Cl	83.2 ±8.2	7.3 ±10.5	0.004	42.9 ±9.7	47.6 ±8.7	0.720
BA			<0.001			0.002

* Outlier in 10° C oral route of administration data previously identified using a Cook's D statistic (see section 4.3.2)

Table IV. Best fit compartmental analysis of plasma oxytetracycline following intra-arterial administration (dose = 100 OTC/kg body weight) in Atlantic salmon held in seawater at 10 ° C. (CI = confidence intervals, $p \leq 0.05$)

		Number of Compartments in Mathematical Model								
		I			II			III		
Time (h)	N	Observed	Estimate	%Dev	Observed	Estimate	%Dev	Observed	Estimate	%Dev
3	4	31.5	16.3	-48.4	31.5	32.4	2.8	31.5	31.5	0
6	4	24.6	15.8	-35.6	24.6	23.7	-3.7	24.6	24.6	0
12	4	14.2	15.0	5.9	14.2	14.3	0.9	14.2	16.2	14.3
24	4	9.1	13.6	49.9	9.1	8.5	-6.1	9.1	9.3	3.2
48	4	5.5	11.0	100.7	5.5	6.8	23.7	5.5	6.1	11.4
96	4	5.5	7.3	34.1	5.5	5.8	6.7	5.5	5.3	-3.2
192	4	4.6	3.2	-29.6	4.6	4.3	-5.6	4.6	4.6	1.2
r^2				0.458			0.994			0.993
CI				(0.43, 1.21)			(2.9, 3.67)			(2.8, 3.56)

Table V. Best fit compartmental analysis of plasma oxytetracycline following intra-arterial administration (dose = 100 OTC/kg body weight) in Atlantic salmon held in seawater at 15 ° C. (CI = confidence intervals, $p \leq 0.05$)

		Number of Compartments in Mathematical Model								
		I			II			III		
Time (h)	N	Observed	Estimate	%Dev	Observed	Estimate	%Dev	Observed	Estimate	%Dev
3	4	39.9	16.3903	-58.92	39.9	39.9	0	39.9	39.9	0
6	4	20.25	15.8382	-21.79	20.25	20.25	0	20.25	20.25	0
12	4	13.35	14.7893	10.78	13.35	10.6151	-20.49	13.35	13.3016	-0.36
24	4	9.0	12.8952	43.28	9.0	8.5259	-5.27	9.0	9.1667	1.85
48	4	5.45	9.8037	79.88	5.45	7.076	29.84	5.45	5.4308	-0.35
96	4	3.35	5.6665	69.15	3.35	4.8962	46.16	3.35	3.5628	6.35
192	4	2.94	1.8931	-35.56	2.94	2.35	-20.19	2.9375	2.9403	0.10
r^2				0.410			0.987			0.999
CI				(0.37, 1.15)			(2.49, 3.27)			(4.56, 5.34)

Table VI. Coefficients of determination (r^2) for the best fit compartmental model analysis of plasma oxytetracycline concentrations following intra-arterial administration to Atlantic salmon (0-192 h; n = 4).

		Number of Compartments in Model			
Subject	Temp.	I	II	III	IV
A	10 ° C	0.6498	0.9952	0.9981	N/A*
B	10 ° C	0.5577	0.9595	0.9795	N/A*
C	10 ° C	0.2595	0.9797	0.9855	N/A*
D	10 ° C	0.2174	0.9863	N/A**	N/A*
Mean	10 ° C	0.4101	0.9875	0.9999	N/A*
E	15 ° C	0.8273	0.9945	N/A*	N/A*
F	15 ° C	0.4391	0.9379	N/A*	N/A*
G	15 ° C	0.6777	0.9917	N/A*	N/A*
H	15 ° C	0.5111	0.9932	0.9994	N/A*
Mean	15C	0.4583	0.9944	0.9931	N/A*

* Not calculated by PKCALC^R due to insufficient data

** Data could not be described by sum of 3 exponentials

Initially, analysis of the data for Atlantic salmon held at 15° C was restricted to the 0-24 h period. There were complications in collecting blood samples due to 1) surgerical problems such as the accidental placement of the catheter in the dorsal sinus instead of the dorsal aorta, or improperly sealed connectors (see section 4.2.2), or 2) aggressive withdrawal of blood samples which could result in the formation of fibrin clots at the tip of the catheter, or pinpoint holes in the catheter walls near the 3-way stopcock. The last two data points on the concentration-time curve (Figure 7) represent the mean of only two fish. Consequently, a conservative data set from the first 24 h of sampling ($n = 4$) was adopted for evaluation of the compartmental analysis and pharmacokinetic parameters for the 15° C intra-arterial route of administration data (Table VII). An open two compartment model was determined to be the best fit, based on percent deviation and coefficients of determination derived from the software program PKCALC^R (Table VIII).

In subsequent evaluations, data was fitted to several compartment models. Values of r^2 for the intravascular administration of oxytetracycline for both temperatures were comparable in the two- and three-compartmental models (Table VI). When a Fisher exact test (80) was performed, there was no significant difference ($p \leq 0.05$) between the slopes of the terminal linear portions of the bi-exponential and the tri-exponential compartment models for the 15° C data. There was, however, a significant difference between the slopes of the bi- and tri-exponential compartment models at 10° C suggesting that the data represented two

Table VII Pharmacokinetic parameters for oxytetracycline following intra-arterial administration to Atlantic salmon (mean ± 1SD; two-compartment model; 0 - 24 h)

Temperature	15 ° C
Route	IA
Weight	481.8 ± 39.98
Number of Fish	4
Dose	100 mg OTC/kg
AUC	533.6 ± 45.1
C_{max}	31.5 ± 6.1
T_{max}	3.0 ± 0.0
VD_{SS}	3.5 ± 0.46
$T_{1/2}$	12.03 ± 1.49
Cl	188.5 ± 17.3

IA	Intra-arterial
Weight (g)	
AUC (µg-h/mL)	Area under the concentration time curve
C_{max} (µg/mL)	Maximum plasma concentration
T_{max} (h)	Time to maximum plasma concentration
VD_{SS} (L/kg)	Apparent volume of distribution at steady state
$T_{1/2}$ (h)	Elimination half-life
Cl (mL/h)	Plasma clearance

Table VIII Coefficients of determination (r^2) for the best fit compartmental analysis of plasma oxytetracycline following intra-arterial administration to Atlantic salmon held at 15° C (n = 4; 0-24 h)

		Number of Compartments in Mathematical Model		
Fish Subject	Temp.	I	II	III
E	15 ° C	0.9202	0.9935	N/A*
F	15 ° C	0.9126	0.8212	N/A*
G	15 ° C	0.9746	N/A**	N/A*
H	15 ° C	0.9452	0.9998	N/A*

* Not calculated by PKCALC^R due to insufficient data

** Data cannot be described by sum of 2 exponentials

distinct phases of elimination (Table V). Since pharmacokinetic data is normally modelled using the least number of compartments, an open two-compartment model was the best description of the elimination kinetics of oxytetracycline (0-192 h) in Atlantic salmon held in seawater at 15° C (Table IV). An open three-compartment model best described the elimination kinetics of oxytetracycline (0-192 h) in Atlantic salmon at 10° C. If there had not been difficulties in sample collection at the later time periods for fish held at 15° C, it seems probable that the three-compartment model would have described the elimination kinetics at both temperatures.

4.4 Discussion

4.4.1 Pharmacokinetic Parameters for IA and PO Routes of Administration

Maximum Plasma Concentration

The maximum plasma concentration (C_{max}) is defined as the peak plasma concentration of drug (7). This study agrees with earlier reports that route of administration influences the maximum plasma concentrations of oxytetracycline in salmonids (33). Oxytetracycline absorption from the gastro-intestinal tract is limited and this was reflected in low C_{max} values following PO administration (Tables I and VII). Maximum serum concentrations of orally administered oxytetracycline have been reported to vary from 0.28 μg/mL in carp (33) to 2.0 ± 0.7 μg/mL in rainbow

trout (13), for fish which had received 34 mg OTC/kg body weight and 75 mg OTC/kg body weight respectively. Interspecies variability, combined with low absorption may account for the low plasma concentrations of oxytetracycline found in this study. However, the first blood samples were not collected until three hours post injection. It may be that peak concentrations of oxytetracycline had been achieved at an earlier time and by the first sampling time of three hours, plasma concentrations of drug were already reduced due to distribution into the peripheral compartments. This would result in an artificially low reading for C_{max}. Since C_{max} was read directly from the disposition curve, the reported values were considered only estimates (Tables I and VII).

Time to Maximum Plasma Concentration

Time to maximum plasma concentration (T_{max})is defined as the time at which the peak concentration of drug occurs in the blood plasma and this can then be used to give an estimate of the absorption rate of a drug in a particular dosage form (8). In this study, T_{max} occurred somewhat earlier than previously reported, 3.0 h post-administration of oxytetracycline for both IA and PO routes of administration (Table I). In comparison, rainbow trout held at 16 ° C receiving a Bacto-agar slurry of oxytetracycline via gastric intubation had a T_{max} which occurred 12 hour post-administration (13), while T_{max} for carp at 20° C has been reported to be 24 h (33). The 12 hour T_{max} reported by Bjorklund and Bylund (13) is comparable to the T_{max}

found during this study for Atlantic salmon held at 10° C (Figure 5). Neither of these two groups of fish (T_{max} = 12 h) had been surgically manipulated prior to gastric intubation. There may be species differences in the rate of absorption, or it may be that the cannulation technique induced physiological changes which accelerated the rate of absorption, distribution and elimination of the drug.

Apparent Volume of Distribution at Steady State

Apparent volume of distribution at steady state (VD_{SS}) is defined as the point when the rate of elimination exactly equals the net rate of distribution into the peripheral compartments. In contrast to other methods for estimating volume of distribution, VD_{SS} is not mathematically dependent upon the rate of elimination. Apparent volume of distribution (VD) relates the concentration of drug in the plasma to the total amount administered (8). However, VD does not provide any information concerning how the drug is distributed. Elevated estimates can occur as the result of sequestering of drug, such as happens with oxytetracycline in bones, scales and pronephros of fish (35). Depressed estimates can also develop as the result of protein-binding. Protein-binding, reported to range from 55% in rainbow trout (13) to 72% in channel catfish, (69). It has been suggested that reported differences in apparent volume of distribution, (33, 35, 69) with values ranging from 1.39 - 2.1 l/kg for oxytetracycline in fish, reflect species differences in tissue

composition, muscle vascularization and ability to accumulate the antibiotic (13, 35, 46).

A large apparent volume of distribution for oxytetracycline was found in this study. Route of administration was shown to have a significant impact on VD_{SS} (Table III). The negative values for VD_{SS}, following an oral administration of oxytetracycline, are a reflection of poor absorption of oxytetracycline in Atlantic salmon. Calculation of VD_{SS} includes the volume of the central compartment, V_c. To calculate V_c, the dose is divided by the concentration in plasma at time 0, but when Cp_o is determined using the software program PKCALC^R the extrapolation, based on the data set, can result in a disproportionately large negative V_c and consequently, VD_{SS}. Chelation of the drug in the bones and scales of the fish have probably contributed to the large VD_{SS} found in this study.

Half - Life of Elimination

The elimination half-life of a drug is defined as the time required for the body to eliminate one-half of the drug "on board". It is derived by measuring the time required for any given plasma concentration of the drug to decline by 50% during the exponential terminal phase of the drug concentration-time profile (7). Values for $T_{1/2}$ are independent of the administered dose since they obey first-order kinetics; but are dependent upon volume of distribution, as well as elimination clearance (8). For fish,

researchers have speculated that a prolonged elimination half-life for oxytetracycline may be due in part to high levels of plasma protein-binding (13, 69) and accumulations of the drug in the pronephros, bone tissue and scales (33, 34).

Statistical analysis of the data indicated that the route of administration had a significant influence on the elimination half-life of oxytetracycline. Oxytetracycline administered intravascularly was eliminated at a rate which was approximately twice as rapid as the elimination rate for OTC that had been administered orally (Table III). $T_{1/2}$ values for an intravascular administration of oxytetracycline to Atlantic salmon held in seawater of 58.3 ± 35.5 h (15° C) to 62.2 ± 14.2 h (10° C) were proportionally similar to $T_{1/2}$ values reported for rainbow trout of 81.5 h at 10° C (15), 89.5 ± 8.7 h at 12° C (35), and 110.0 h at 9° C (44). $T_{1/2}$ for orally administered OTC ranged from 72.5 ± 8.4 h in Atlantic salmon held in seawater at 10° C to 171.6 ± 89.6 h for Atlantic salmon held in seawater at 15° C. However, since the data for fish held at 10° C is based on only two fish, $T_{1/2}$ may be a spurious value. When the previously identified "outlier" fish data was included, then $T_{1/2}$ would be similar to the previously reported elimination half-life in freshwater rainbow trout at 10° C, of 146.4 ± 38.4 h (12). It was not possible to draw any firm conclusions about the elimination half-life for orally administered Atlantic salmon.

The primary route of elimination for oxytetracycline is apparently via biliary excretion, with some drug eliminated via the renal route (46, 69). Each cycle of

entrohepatic re-circulation will result in some systemic loss of oxytetracycline. In fresh-water conditioned fish, $T_{1/2}$ for OTC might be expected to be prolonged in comparison to seawater fish. The higher concentrations of cations in the gastro-intestinal tract of seawater conditioned fish would reduce the concentration of oxytetracycline that would be re-absorbed from the gastro-intestinal tract. More detailed studies, comparing Atlantic salmon held in fresh and in seawater will need to be undertaken in order to clarify whether the differences in $T_{1/2}$ were due to differences in total elimination processes or the result of the amount of divalent cations present in the environment. In addition, there may be species differences in total elimination processes, or the reduced $T_{1/2}$ may reflect differences due to seawater acclimation instead of the fresh water conditions which the rainbow trout in the previous studies were maintained.

Plasma Clearance

Clearance may be defined as a volume of reference fluid (plasma) cleared of a drug by various elimination processes per unit time (7). As with the other pharmacokinetic parameters reported in this study, route of administration was shown to significantly influence clearance (Table III). Consistent with the results for $T_{1/2}$, clearance was very rapid in orally dosed fish.

Previous researchers have suggested that, in fish, tetracyclines are removed from the blood by the liver, concentrated, excreted by the biliary route and then reabsorbed in the upper small intestine resulting in an entrohepatic circulation (13, 18, 21, 46, 69). The secondary peaks which occurred at 24 h in the mean elimination profiles for the orally dosed Atlantic salmon at 15 ° C suggest that entrohepatic re-circulation may have been occurring (Figure 7). While the 10° C oral route data does not show a defined secondary peak, the presence of a plateau (12-24 h) suggests that there may have been too few animals used in this experiment for the reliable detection of a second peak. Further evidence for entrohepatic recirculation are implied in the percent deviation (Tables IV and V) for orally dosed fish between the 12 h and the 48 h time frame.

A similar peak was observed in the low dose (100 mg OTC/kg body weight) IP Atlantic salmon in the pilot study. In that study, the primary peak occurred at 3 h suggesting that re-circulation, in the low dose (100 mg OTC/kg body weight) IP group, may have occurred at about 12 h (Figure 5). A second peak may have occurred after the 36 h termination time for the study. There was no similar pattern observed for the high dose IP group. It is possible that the higher initial concentration of drug may have resulted in a prolonged absorption profile. If the sampling times of the trial had extended past the 36 h, a similar elimination profile might have been observed in the high dose IP group of Atlantic salmon.

The presence of a secondary peak may also indicate re-distribution of the drug from the well-perfused tissues, such as kidney, to more poorly perfused tissues, such as muscle. Baggot (7), suggests that the most effective way to develop an outline of the distribution pattern is by a whole-body autoradiography study, such as was undertaken by Ingebrigsten *et al.* (46), using rainbow trout. High levels of radio-labelling occurred in the bile as well as in the mucosal lining of the intestinal tract suggesting that entrohepatic circulation of oxytetracycline was occurring in rainbow trout. Further research using radio-isotope labelling of OTC might provide more conclusive evidence for an entrohepatic re-circulation pattern in Atlantic salmon.

Area Under the Curve and Bioavailability

Bioavailability is a term used to indicate the extent to which a drug administered as a particular dosage enters the systemic circulation in its active form. By definition an intravascular administration of drug represents 100% systemic availability (7). An oral administration of drug will express less than complete systemic availability. Using the formula, AUC_{PO} obtained following an oral administration of oxytetracycline divided by the AUC_{IA} obtained following an equal dose of oxytetracycline administered intra-arterially, it was possible to calculate systemic bioavailability for oxytetracycline at 10° C as 1.5% (confidence intervals of 0.8%, 2.7%), and at 15° C as 7.4% (confidence intervals of 4.1%, 13.6%).

The apparent bioavailability for the oral administration of oxytetracycline was low in this study. Comparing the corresponding area under the concentration-time curves (AUC) for the intravascular and the oral route of administration demonstrated that route of administration had a significant impact on bioavailability (Tables II and III). Previous reports of 7-9% apparent digestibility in rainbow trout (21), 2.7% per os bioavailability in channel catfish (69), 0.6% per os in carp (35), and 5.6% per os in rainbow trout (13) indicate that oxytetracycline is poorly absorbed by teleosts. Oxytetracycline has the ability to form complexes with divalent and trivalent cations present in the environment or in feed (69), and this reduces its bioavailability (53). Marine teleosts are hypo-osmotic relative to their environment and must drink seawater continuously, consequently under seawater conditions the stomach contents may be considered to be slightly modified seawater (57). The poor bioavailability for oxytetracycline may be partially explained by chelation with divalent ions present in the gastro-intestinal tract. Similar results have been reported in mammalian systems, the bioavailability of oxytetracycline has been reported to be conditioned by the presence of divalent cations (41). In humans the consumption of milk and tetracycline medications, at the same time, is contra-indicated.

4.4.2 Pharmacokinetic Parameters at Different Temperatures

Maximum Plasma Concentration and Time to Maximum Plasma Concentration

The analysis by general linear model indicated that C_{max} would not exhibit a significant temperature dependence. Factorial analysis, however, indicates the presence of simple temperature effects on C_{max}, a function of the route of administration utilized. It appears that T_{max} is somewhat less affected by temperature following an inter-arterial route of administration than following an oral route of administration. Absorption was rapid at both temperatures (T_{max} = 3.0 h), although there was large animal to animal variation in the orally dosed fish during the initial sampling times (0-24 h). This is similar to previously reported differences in uptake of OTC from medicated feed by Chinook salmon held in seawater (3). The rate and extent of absorption and metabolism, and gastric emptying rate are all factors which are temperature dependent (12, 26, 48, 74). Both general linear model and factorial analysis indicate that temperature affects T_{max}.

Apparent Volume of Distribution at Steady State

Apparent volume of distribution at steady state, VD_{SS}, includes both the volume of the central compartment (plasma) and the peripheral compartment(s). In this study the VD_{SS} was very large (5.95-7.5 l/kg) suggesting extensive extravascular

distribution. While the general linear model (Table III) did not indicate any significant temperature effects, factorial analysis demonstrated interactions which suggested that there were simple temperature effects on the apparent volume of distribution of oxytetracycline from the plasma of Atlantic salmon. Lambs *et al.* (55), showed that oxytetracycline tends to chelate to the cations present in both bones and scales. This would reduce the "free" drug while generating a large VD_{SS}. Residual oxytetracycline sequestered in bone and scales, where the rate of calcium turnover is slow, is unlikely to be susceptible to the influences of temperature. Koenings *et al.* (51), were still able to identify free-ranging sockeye salmon which had been marked with OTC nine months earlier. Oxytetracycline, sequestered in skin and other sites where the calcium turnover was rapid, is apparently replaced shortly after cessation of medicated feed. Researchers thought that the calcium in the skin was either replaced, or used to meet short term metabolic needs (51). Temperature would likely influence the VD_{SS} of OTC ("free" and OTC-calcium complexes at sites of rapid turnover) through rate changes in the rate of metabolic activity.

Elimination Half -life

The elimination half - life for orally dosed Atlantic salmon of 171.6 ± 89.6 h at 15° C was higher than previously reported values for rainbow trout, 74.9 ± 8.2 h (13) at 16° C, but the $T_{1/2}$ (10° C) of 72.5 ± 8.4 h was slightly lower than previously reported values of 115.2 ± 31.2 h to 146.4 ± 38.4 h at 10° C for rainbow trout (12,

66). It has been suggested that temperature has a profound influence on $T_{1/2}$ (13, 66). Statistical analysis of the data from this study using GLM indicated no significant influence on the elimination half-life due to temperature. However, factorial analysis reveals the presence of significant interactions between the route of administration and temperature on elimination half-life. Following an inter-arterial route of administration there was a slight affect on T_{max} due to temperature, but T_{max} was significantly influenced by temperature following the per os route of administration. The rate and extent of absorption, metabolism and elimination are all factors which are temperature dependent (12, 26, 74).

Clearance

Clearance refers to the volume of plasma cleared of drug per unit time and is a function of both volume of distribution and the rate constant of elimination. Oxytetracycline has a large volume of distribution, but since much of the drug is apparently chelated to cations present in bones and scales, with a low rate of calcium turnover, VD_{SS} was not significantly affected by temperature. $T_{1/2}$ was affected by temperature, but most of the effect was only observable when oxytetracycline was administered via the oral route. Since temperature did not significantly influence either of these parameters following an intravascular administration of drug, it would not be expected to influence the rate of clearance. Both GLM and factorial

statistical analysis, showed that clearance was not affected by the temperature in which the Atlantic salmon were held.

Area Under the Curve and Bioavailability

Fish are poikilothermic and at higher temperatures absorption, distribution and elimination should occur at a greater rate as the result of higher metabolism (13, 35). Statistical analysis using the general linear model and factorial analysis indicated that temperature would have no significant effect on the area under the curve (Table III). Since area under the curve is used to calculate bioavailability, temperature should not influence bioavailability either. However, calculating BA using IA and PO data was difficult since the data were highly skewed. To compensate for this the variance was stabilized with a logarithmic transformation. When this was done temperature became a significant predictor for bioavailability (Table III). As previously discussed in section 4.4.1, absorption and bioavailability of oxytetracycline is low ($<10\%$ in carp, trout, catfish and in this study). The decreased bioavailability of oxytetracycline absorption at 10° C may reflect temperature influences upon the composition and fluidity of the mucosal lining of the gastro-intestinal tract and of biological membranes (29, 45).

4.4.3 Compartmental Analysis: Compartmental analysis is a mathematical approximation of a real system under study. It consists of a series of inter-connected

compartments into which drug is absorbed, distributed and eliminated. Drug absorption, distribution and elimination, expressed graphically as a plasma concentration-time curve, may be described by one exponential curve (one compartment model), or by the sum of two or more exponential curves (two or more compartments). The compartments of the model do not necessarily have any physiological or anatomical correlation.

In order to determine the model that best describes the observed plasma concentration-time profiles, the linear terminal portion (elimination phase) of the disposition curve is used. In biological systems, the most commonly observed pharmacokinetic model is multi-compartmental, the result of differential diffusion of drug in tissues. To calculate kinetic parameters for a two-compartment model, the elimination or second (β) phase is utilized. In a three - compartment model the second elimination (γ) phase is used in the calculations.

Previous researchers have described both open two compartment (13) and an open three compartment (35) models for the elimination of oxytetracycline in rainbow trout. In this study, an open two compartment model was determined to be the best fit for the elimination kinetics of oxytetracycline from Atlantic salmon held in seawater at 15° C. Based on the coefficient of determination, there was some indication that a third compartment might be present (Table IV), but a Fisher exact test indicated no difference between the slopes of the terminal linear portions of the

two- and three- compartment models. It appeared that either the second elimination phase behaved in a manner similar to the first elimination phase, that is the rate of elimination was so similar as to be in indistinguishable, or the limited data points in the later sampling obscured the third compartment. Biological variability in the absorption of oxytetracycline has been reported (3) and it is possible that elimination of oxytetracycline exhibits the same type of individual variability.

The same approach that was used for evaluation of the 15° C data, the coefficient of determination, percent deviation and a Fisher exact test, was utilized to evaluate the data from Atlantic salmon held at 10° C (Table V). An open three-compartment model was determined to be the best description for the plasma elimination kinetics of oxytetracycline from Atlantic salmon held in seawater at 10 °C. Thus, the plasma elimination kinetics of oxytetracycline in Atlantic salmon held in seawater and at different temperatures would appear to be tri-exponential. Inconclusive evidence from the 15° C trials, will make it necessary for more research to be completed in order to accurately determine the correct compartmental analysis for Atlantic salmon.

4.4.4 Conclusions

The plasma pharmacokinetics and bioavailability of oxytetracycline for Atlantic salmon held in seawater at 10° C and 15° C were determined in this study. An open

three-compartment model best describes the elimination kinetics of oxytetracycline in Atlantic salmon held in seawater at 10° C, while an open two-compartment model was adequate to describe the elimination kinetics at 15° C. The use of a two-compartment model may not have been an accurate description of the plasma pharmacokinetics of oxytetracycline in Atlantic salmon held in seawater at 15° C, as there were indications that a third elimination compartment might have been present. Not unexpectedly, route of administration was shown to be a significant predictor for pharmacokinetic parameters of oxytetracycline in Atlantic salmon. In general, temperature has been identified as having an influence on pharamcokinetic parameters. In this study, temperature had a significant affect on bioavailability but the other pharmacokinetic parameters exhibited only simple temperatures affect based on the route of administration. Oxytetracycline which had been administered by per os was more susceptible to temperature affects than drug which had been administered intra-arterially. More research is needed in order to clarify the specifics of temperature effects in marine teleosts.

Based on the low bioavailability found in this study, the therapeutic use of oxytetracycline in salmonids held in seawater may be questionable, especially when combined with the immunosuppressive effects of tetracyclines in fish (34) and the potential for environmental loading (14).

5.0 GENERAL DISCUSSION

The research objectives indicated in Chapter One have been accomplished as described in chapters Two, Three and Four. Much of the research, such as finding significant differences ($p \leq 0.001$) between the oral and the intraperitoneal, and the oral and intravascular ($p \leq 0.05$) routes of administration, represented a confirmation of conventional theories, based on fresh water species. Some specific issues arose throughout the project.

5.1 Methods of Evaluating Chemotherapeutics in Fish Plasma

The problems associated with methods for the assay of oxytetracycline in body fluids and tissues of aquatic species were discussed in chapter Two. Detection of drug residues is limited only by the sensitivity and the specificity of the analytical methods (37). In spite of the popularity of HPLC techniques for pharmacokinetic studies of oxytetracycline in aquatic species (11, 27, 47, 63, 65), the AOAC microbiological assay for oxytetracycline in feeds (84) was adapted for use throughout this study. HPLC techniques are specific, that is they separate the parent drug and its metabolites, but previous researchers have suggested that the metabolites are frequently uncharacterized in pharmacokinetic studies. In the case of oxytetracycline, there appears to be limited or no occurrence of metabolism of oxytetracycline by fish species (12, 21). In mammalian systems, there is general agreement that the

tetracyclines do not undergo metabolic biotransformation (73). If true for fish, then the concern for specificity may be unwarranted, and the use of HPLC techniques could be reduced, or eliminated, in pharmacokinetic investigations of oxytetracycline.

In order to ensure physiological relevance, any pharmacokinetic model should provide a description of the free (available) antibiotic concentration in the pertinent tissues (53). Any measure of oxytetracycline by HPLC will generate the total concentration of the drug, and the protein-bound oxytetracycline, reported to range from 55% in rainbow trout (13) to 72% in channel catfish (69). Neither of these forms are readily available to combat a bacterial infection and therefore, do not have physiological relevance for the fish until such time as they do become available. While there is some suggestion that HPLC techniques are unnecessary for the assay of oxytetracycline in the plasma of aquatic animal species, it would be worthwhile for further researchers to confirm the presence or absence of oxytetracycline metabolites after oral dosing and to complete a comparative study between the AOAC microbiological assay developed in this project and an HPLC approach for the analysis of oxytetracycline in fish plasma to determine the best method for routine evaluations.

5.2 Pharmacokinetics and Bioavailability of Oxytetracycline

The absorption and elimination of oxytetracycline was studied at 10° C and 15° C following intravascular and oral administration to Atlantic salmon held in reconstituted seawater. An open three-compartment model best describes the elimination kinetics of oxytetracycline in Atlantic salmon held in seawater at 10° C, while an open two-compartment model described the elimination kinetics at 15° C. Since there was some evidence for the presence of a second elimination phase, the two-compartmental model for the elimination of oxytetracycline from the plasma of Atlantic salmon held at 15° C may be inaccurate. It appears likely that the best description for plasma elimination of oxytetracycline in Atlantic salmon held in either 10° C or 15° C should be a three compartmental model, but additional research is required to clarify whether the bi- or tri- exponential model is the correct explanation in Atlantic salmon held at 15° C.

Previous researchers (18, 21, 46, 69) have suggested entrohepatic re-circulation of oxytetracycline in fish. HPLC data from Iversen *et al.* (47), also indicated a secondary absorption of oxytetracycline occurred in rainbow trout, but whereas in Atlantic salmon the second absorption peak occurred between 12 and 24 h, in the Iversen study with rainbow trout the secondary absorption occurred at approximately 24 - 36 h. Further research using radio labelling of OTC might

provide more conclusive evidence for an entrohepatic re-circulation pattern in Atlantic salmon.

Estimates for bioavailability of drugs in Atlantic salmon are generally low (41), and this investigation with oxytetracycline is not an exception. Experimental results indicate a low apparent bioavailability for oxytetracycline in Atlantic salmon held in seawater. The apparent bioavailability for oxytetracycline (0 - 192 h) was 1.5% at 10° C and 7.4% at 15° C. Previously reported values for oxytetracycline bioavailability (13, 21, 35, 69) in fresh water teleosts were also very low (0.6% to 5.6%). The similarity between the bioavailabilities of oxytetracycline in fresh water and seawater suggests that there is no apparent differences between fish held in fresh water versus seawater. But each of the previous authors administered the oxytetracycline with feed, while a slurry was used in this experiment. Chelation with divalent ions normally present in feeds may account for the reduced bioavailability in the previous studies with fresh water species. In order to more fully understand the effects of bioavailability of oxytetracycline in seawater versus fresh water aquatic species, a comprehensive evaluation would have to be undertaken comparing Atlantic salmon or rainbow trout (of the same cohort), one group conditioned to seawater while the other group was held in fresh water.

5.3 Elimination Kinetics and Bioavailability of Oxytetracycline at Different Temperatures

In general, the absorption, distribution and excretion of oxytetracycline is reported to be temperature-dependent (13, 28, 35, 48, 74). It had been expected that there would be a near 10% increase in the rate of elimination with each 1° C increase in water temperature (26), which meant that the absorption and excretion of oxytetracycline should have occurred at a much greater rate at the higher temperature. In this study, statistical analysis demonstrated that temperature had a significant influence on bioavailability, but apparently did not influence the processes of distribution and excretion. The lack of significant influences due to temperature (GLM method) is the consequence of the effect being averaged out over time. Factorial analysis, did indicate that there were simple temperature effects occurring with C_{max}, T_{max}, VD_{SS} and $T_{1/2}$. The intra-arterial route of administration appeared to be less susceptible to the influence of temperature changes than the per os route of administration.

The primary reason for the administration of chemotherapeutics is control of bacterial diseases. For this to be accomplished therapeutic levels must be achieved at the site of infection. The Minimum Inhibitory Concentration (MIC, C_{50}) value, defined as the lowest concentration of drug required to inhibit 50% of the bacterial growth, has been reported to be 1.0 µg OTC/mL at 10° C and 20° C for the causative

agent of furunculosis *Aeromonas salmonicida* (25). It is generally accepted that the concentration of the antibiotic must exceed the MIC by five times. In this study, plasma concentrations of oxytetracycline in Atlantic salmon did not surpass plasma concentrations at either 10° C or 15° C, which is different from results reported by previous researchers (13, 21). The fish in this study received a single administration but, in the preceding studies the fish were evaluated under field conditions and so they received medicated feed for 10-12 days. There was a cumulative increase in the plasma concentration of drug over time. Increased plasma concentration of oxytetracycline would be achieved if the drug had been administered for 10 days. Confirmation of plasma concentrations of oxytetracycline in Atlantic salmon could be verified by conducting a 10 day feeding trial using OTC-medicated feed and evaluating plasma concentrations for another 10 days after medicated feeding had ceased.

Based on the low bioavailability found in this study, the therapeutic use of oxytetracycline in salmonids in seawater may be questionable, especially for animals held at 10° C. When combined with the reported immunosuppressive effects of tetracycline in fish (35) and the reported increase of oxytetracycline resistant bacteria in effluents from OTC treated fish farms (6), and in the sediments under aquaculture sites (13, 42), a re-evaluation of the use of oxytetracycline in the treatment of fish diseases may be warranted.

5.4 Future Research

In summary, there are several areas in which future research would be beneficial.

1) In order to confirm the presence, or absence, of OTC metabolites and evaluate the recovery of OTC from fish plasma, a comparison of the modified microbiological assay, developed in this study, with HPLC methods should be undertaken.

2) Additional research should be undertaken to determine whether the elimination kinetics of OTC in Atlantic salmon held in seawater at 15° C, is best described as a bi- or tri-exponential model.

3) A comparison between Atlantic salmon, or rainbow trout (of the same cohort), one group conditioned to seawater and the other group held in freshwater would clarify the effects of salinity on the bioavailability of OTC.

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APPENDIX A

Fish Marking Via Cold-Branding

A reliable method of marking fish is an asset when undertaking ecological, nutritional, genetic or pharmacological research. The criteria for the ideal mark should include the maximum amount of information with the minimum effect on the physiology, or behaviour of the fish. Cold-branding is rapid, inexpensive and, when done properly, will result in less than 1% mortality.

The cold-branding unit was based on the design previously described in Module G-2 [2]. Modifications included 1) housing of the unit in a plastic canister, and 2) welding a hollow metal tube into position so that it passed through the styrofoam and the welded steel reservoir (about 40 cm above the bottom) to the opposite reservoir wall (see Figure 8). Heat transference (loss) between the 5 litres of liquid nitrogen (-196 ° C) contained in the welded steel reservoir and the branding iron readily occurred when the branding iron was inserted into the hollow tube. The branding iron consisted of a single copper bar mounted on the tip of a plastic grip screwdriver.

Prior to branding, fish are anaesthetized with MS-222 (Syndel Laboratory Ltd. Vancouver, B.C.) 100 mg/L, placed onto a water-saturated section of foam and held

[2] Module G-2 (Marking and Tagging of Fish) 1977 in Aquaculture Technician Training Manual, Huntsman Marine Laboratory, St. Andrews, N.B.

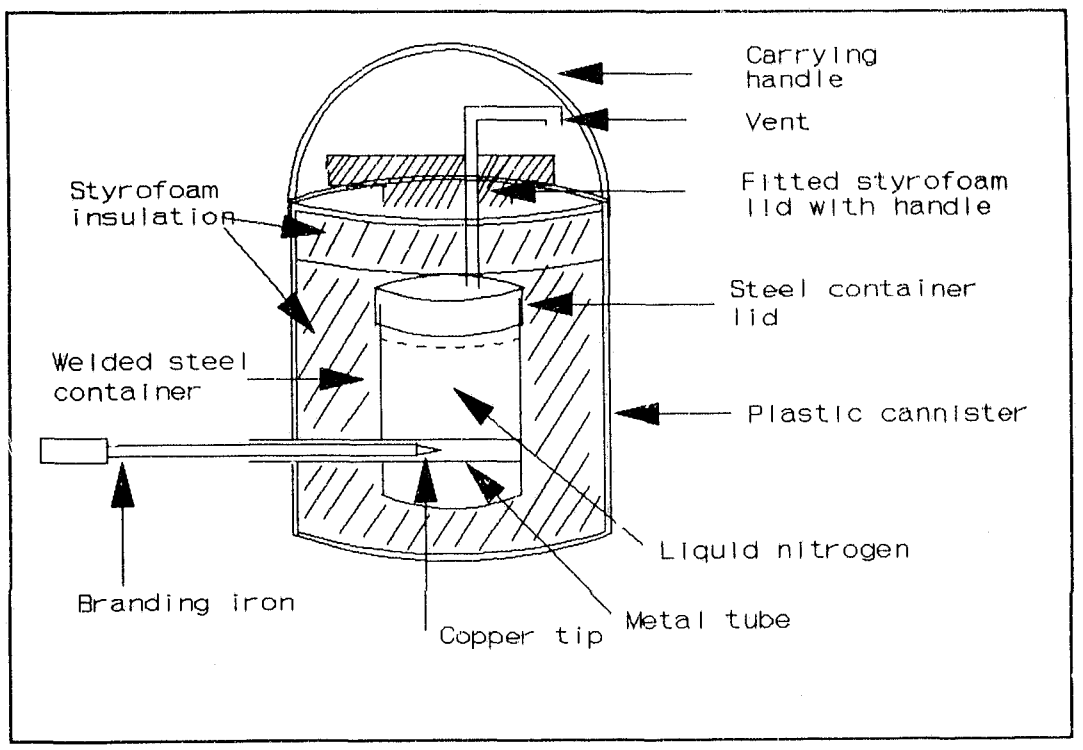

Figure 8. Freeze-branding unit with branding iron

securely. Branding occurs by firmly applying the previously cooled branding iron to the epidermis for 2 - 7 seconds, depending on species. Up to seven single bars can be applied before the branding iron must be re - cooled. The anterior dorsal surface next to the cranium and right or left of the dorsal fin tend to be the most readily visible through the water column. Other regions which may be branded include left or right operculum, and left or right caudal peduncle. Brands should be placed above the lateral line for best visibility through the water column.

The brand design is very important in that identifying individual fish is easier with a simple bar design as opposed to the use of symbols, numbers or V/Z shapes. Combinations of vertical and horizontal brands, right and left brands, number of brands or combinations of sites of brands can be used to increase the number of individuals that can be identified.

Immediately following the cold brand a white frozen layer of mucus and epithelial tissue was observed. Within 30 minutes the lesion will change to a dark outline bordered by a light 'halo'. Initially, the brand consists of a black line of melanophores where the granule control system has been affected but over time, the outline of the brand becomes more diffuse. This phenomenon is typical of healing tissues in salmonids. Water temperature will also affect the length of time that the brand is easily read. The higher the water temperature, the more rapid the healing

process and the shorter the length of time during which reliable readings of the brands can occur.

Cold-branding has been criticized for two reasons, 1) length of time which the brand is easily visible is a function of species and temperature at which the animals are held, and 2) the high variability in the quality and subsequent recognition of the brand. Legibility of the brand appears linked to physiological changes (black pigmented line at brand site transforms to a progressively more diffuse outline) which occur at approximately six - twelve months post-branding. Application time for Atlantic salmon has been reported as two second contact time (54), and three second contact time (36). Of the three cold-branding contact times (2, 3, 7 seconds) evaluated in this experiment, the two second brand had faded after a month at 10° C; the seven second branded fish showed evidence of tissue necrosis at the contact site. A three to five second cold-brand was identified as the best selection for Atlantic fish held at 10° C in seawater.

APPENDIX B

Equations Used by PKCALC[R]

1) Sample mean

$$\overline{C} = \Sigma \frac{C}{n}$$

C = concentration

n = number of observations

2) Sample Standard Deviation

$$SD = \sqrt{\frac{\Sigma(C - \overline{C})^2}{n - 1}}$$

3) Standard Error of the Mean

$$SE = \frac{SD}{\sqrt{n}}$$

4) Coefficient of Variation as a Percent

$$CV = \frac{SD}{\bar{C}} \cdot 100$$

5) Interval AUC by Linear Trapezoidal Method

$$\left[AUC_{Lin} \right]_{t_{i-1}}^{t_i} = (t_i \cdot t_{i-1}) \times \frac{(C_i + C_{i-1})}{2}$$

t = time sample was collected

6) Total AUC

$$AUC_{0-\infty} = \Sigma \left[AUC \right]_{t_{i-1}}^{t_i} + C_T^* / \gamma_Z$$

AUC = Area under the Curve

C_T^* = estimated concentration at the time (T) of the last utilized sample

γ_Z = slope of the regression line representing the terminal elimination half-life

7) Total Clearance

$$Cl = \frac{F.D}{AUC_{0-\infty}}$$

F = formulation availability

D = dose

8) Volume of Central Compartment

$$V_c = \frac{D_N}{C_0^*}$$

C_0^* = sum of the zero-time intercepts obtained from extrapolation of the linear regression lines

9) Volume of Distribution at steady state

$$VD_{SS} = V_c \frac{K_{12} + K_{21}}{K_{21}}$$

K = elimination constant

10) Elimination Half-life

$$T_{\frac{1}{2}} = \frac{0.693}{\beta}$$

β = overall elimination rate constant