

**Florfenicol and Calcein: Update on the Completion of an Initial Approval for
Use of Florfenicol-Medicated Feed in Aquaculture and on a
New U.S. Fish and Wildlife Service Investigational New Animal Drug**

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In the United States (U.S.), federal, state, tribal, and private aquaculturists rear more than 100 species of aquatic animals. Unfortunately, there are few U.S. Food and Drug Administration (FDA)-approved drugs that can legally be used to treat these animals when disease outbreaks occur, or when there is a need to anesthetize or mass mark fish. Consequently, in 2003, the U.S. Fish and Wildlife Service (Service) established the Aquatic Animal Drug Approval Partnership (AADAP) Program, the mission of which is to ensure continued progress towards obtaining FDA-approved new animal drugs for use in aquatic species.

The AADAP Program will provide the expertise and funding means to assist federal, state, tribal and private aquatic animal culturists meet their animal disease management needs. The AADAP will focus efforts on a prioritized list of candidate drugs that will be available to all culturists and qualified animal health professionals. The AADAP is a broad, partner-based national program, and will help to lead a coordinated effort to generate data, analyze results, compile final study reports, disseminate information and data, and manage or assist with all other aspects of requisite data submissions to FDA in support of new animal drug approvals for aquatic species. The AADAP will also help coordinate Service activities to ensure hatchery compliance with EPA discharge regulations as they relate to drugs and chemicals. The AADAP builds on a long-standing partnership between the Service and over 50 federal, state, tribal and private agencies or organizations. AADAP will continue to administer the Services highly successful National INAD Program, whereby both Service and non-Service participant facilities are allowed to use needed drugs closely supervised and monitored under compassionate INAD exemptions. Currently, the AADAP administers 14 individual INADs, including 9 therapeutants/antimicrobials, 1 anesthetic, 2 spawning aids, and 2 marking agents.

Although AADAP is involved in drug approval activities related to numerous potential new aquaculture drugs, two compounds of particular interest to the aquaculture community are the use of (1) calcein for the mass-marking of larval fish, and (2) the use of florfenicol as a broad spectrum antibacterial agent. This paper describes the current status of ongoing efforts to complete new animal drug approvals for calcein and florfenicol.

Florfenicol update

Bacterial diseases remain a major problem in aquaculture and account for significant losses of fish (Clark and Scott 1989; Frerichs and Roberts 1989; Bjorndal 1990). Although the importance of environmental conditions (McCarthy and Roberts 1980; Haastein 1988; Munro and Roberts 1989) and the value of effective vaccines, where available (Ellis 1989), are acknowledged, antimicrobial therapy presently has an important role to play in aquaculture (Klontz 1987; Alderman 1988).

Florfenicol is a potent, broad-spectrum antibacterial agent with bacteristatic properties (Horsberg et al. 1996). It is a fluorinated analogue of thiamphenicol and is also similar in structure to chloramphenicol, both of which have been used as broad-spectrum, veterinary antibiotics (Nagata and Oka 1996). Florfenicol has great potential for treatment of infectious diseases, and because of its high potency and safety to humans, it could become an important drug in veterinary medicine, especially with respect to animals used by humans for food (Powers et al. 1990).

AquaflorTM is an aquaculture premix containing 500 g florfenicol per kg of premix and is available only from Schering-Plough Animal Health (SPAH; Union, NJ). AquaflorTM is approved for use in Canada to control mortality in Atlantic salmon *Salmo salar* caused by furunculosis (causative agent *Aeromonas salmonicida*). In Canada, the approved treatment regimen is to administer 10 mg florfenicol/kg fish/d for 10 consecutive days. AquaflorTM is also approved for use in the United Kingdom, Norway, Israel, Spain, and Chile to control mortality in a variety of fish species caused by a variety of pathogens. In addition, NuflorTM, SPAH's injectable form of florfenicol, is approved for use in most major markets worldwide, including the U.S., for cattle and swine for a variety of disease indications.

For the past several years, the Service has had a compassionate INAD exemption for AquaflorTM for use to control mortality in a variety of fish species caused by pathogens susceptible to florfenicol. Because of the success of the aquaculture INAD, and the success of florfenicol products in other markets, SPAH seriously investigated gaining FDA-approval of AquaflorTM for use in aquaculture in the U.S.

A new animal drug approval is dependant upon FDA acceptance of data to complete the following technical sections: (1) Product Chemistry, (2) Efficacy, (3) Target Animal Safety, (4) Human Food Safety (an extensive technical section that includes toxicology tests, identification of the marker residue, analytical methods development, residue depletion studies, and storage stability), and (5) Environmental Safety. FDA is often asked whether data used to gain approval of a drug for use in aquaculture in other countries, or data used to gain approvals in the U.S. for uses in food animals other than fish can be used to fulfill all or parts of technical sections required for approvals in aquaculture. Fortunately, in some cases, data generated to recent FDA standards in the U.S. or abroad can be used to fulfill some of the requirements. Fortunately, such

was the case with some of the data that had previously been generated by SPAH for other approvals.

Toxicology data used to gain FDA approvals for florfenicol use in cattle and swine, and environmental safety data generated in the U.S. and Europe to recent U.S. standards, were accepted by FDA for use in aquaculture. Furthermore, FDA required no new studies from SPAH for either technical section. In addition, antimicrobial resistance tests from florfenicol use in the U.S. in other domesticated animals, using pathogen strains found in the U.S. and following National Committee for Clinical Laboratory Standards, were also found to be acceptable and confirmed that florfenicol had no effect on human gut flora or on human pathogens. Because of the time and high cost associated with completing the above-mentioned technical section components, FDA acceptance of this data was a crucial decision-point for SPAH. Upon acceptance of the data, the sponsor decided it was prudent to proceed with efforts to gain FDA approval of Aquaflor™ for use in aquaculture.

Over the past several years, substantial progress has been made to complete the remaining technical sections required for an initial approval of Aquaflor™. At this time, SPAH is focusing efforts on trying to gain an approval for limited use of Aquaflor™ in freshwater-reared salmonids and catfish. Nearly all technical section completion requirements for these approvals have been completed, and most have been accepted by FDA. The status of these technical sections specific to salmonids are described below:

Efficacy - Efficacy data generated to gain approvals of Aquaflor™ for aquaculture in other countries were submitted to FDA in hopes they would fulfill U.S. efficacy technical section requirements. However, these data were not accepted because FDA requires that efficacy studies be conducted using pathogen strains found in the U.S. Therefore, AADAP took lead responsibility to conduct field trials to demonstrate the efficacy of florfenicol to control mortality in freshwater-reared salmoides caused by coldwater disease (causative agent *Flavobacterium psychrophilum*), furunculosis (causative agent *A. salmonicida*), and columnaris (causative agent *F. columnare*). AADAP conducted several trials that demonstrated the effectiveness of florfenicol to control mortality in steelhead *Oncorhynchus mykiss* and cutthroat trout *O. clarki* caused by coldwater disease. As a result of FDA acceptance of these studies, the technical section for the control of mortality in all freshwater-reared salmoides caused by coldwater disease has been completed. Studies have also been accepted by FDA that demonstrate the effectiveness of florfenicol to control mortality in coho salmon *O. kisutch* caused by columnaris and furunculosis. FDA has informed AADAP that the technical sections for columnaris and furunculosis will be complete following submission of one additional FDA-acceptable study for each disease indication on a salmonid species other than coho salmon.

Target animal safety - The target animal safety technical section for freshwater-reared salmonides has been completed following FDA-acceptance of a study on rainbow trout *O. mykiss* conducted by researchers at the University of Sterling in Scotland.

Residue depletion - The residue depletion portion of the human food safety technical section is nearing completion following submission of a Final Study Report (FSR) that described results from a study on rainbow trout conducted at the University of Sterling in Scotland. The FSR was submitted to FDA on November 24, 2003. At this time, SPAH is anticipating that it will take 12 - 15 months for FDA to review this report, inform SPAH of possible deficiencies identified in the study or report, and for SPAH to resolve the deficiencies.

Feed assay method transfer study - A study has been completed, submitted, and accepted by FDA. Feed assays may now be performed by qualified scientists at the U.S. Geological Survey's Upper Midwest Environmental Sciences Center (LaCrosse, WI) and at Eurofins Scientific (Memphis, TN). AADAP will contract with Eurofins Scientific to assay feed used in future efficacy studies in support of an expanded approval for florfenicol.

Product chemistry - FDA acceptance of the product chemistry technical section and other parts of a new animal drug application that are typically the responsibility of the sponsor is a good indication of SPAH's high level of commitment to gain an initial approval for florfenicol. To date, SPAH has completed the following: (1) Chemistry and Manufacturing Controls, which is basically the process of taking the manufacturing of the drug in the lab to bulk production of the premix formulation; (2) Other Pertinent Information (OPI), which is data, reports, or manuscripts that were not used directly in support of the approval of florfenicol, but may indirectly support its approval, have been assembled and submitted to FDA; (3) Updating the Freedom of Information (FOI) Summary is regularly updated by SPAH each time a part of a technical section has been completed. SPAH has committed to continue updating the FOI Summary until as broad a label claim as possible has been achieved. SPAH has also committed to properly label the new product and to submit to FDA a New Animal Drug Application (the last step in the process) in a timely manner.

Progress has moved along relatively quickly with Aquaflor™, in large part because SPAH has directed considerable resources towards gaining an initial approval for use of Aquaflor™ to control mortality in all freshwater-reared salmonides caused by coldwater disease (at a dosage of 10 mg florfenicol/kg fish/d for 10 d). The sponsor is hopeful that the initial approval will be granted by the end of CY2004. In addition, as soon as AADAP successfully completes planned field efficacy studies in which the disease indication is either columnaris or furunculosis and submits resulting FSRs to FDA, then SPAH will expand the initial approval request to include these disease indications.

Individuals in the public sector that are involved with the approval of Aquaflor™, as well as those involved with approvals of aquaculture drugs in general, are extremely pleased

with how quickly SPAH has progressed towards the impending initial approval of Aquaflor™. This rapid progress was made possible, in part, to (1) the high level of sponsor engagement, (2) the amount of data generated for other approvals that were acceptable to FDA for an aquaculture approval, and (3) a coordinated effort by the sponsor and researchers in the public sector to complete outstanding technical sections. The process used by SPAH should be considered as a workload model for aquaculture drugs, and is recommended to others interested in gaining FDA drug approvals.

Calcein update

Over the years, numerous methods have been developed to mark or tag individual fish prior to stocking fish for recreational, mitigation, or enhancement purposes. While these methods have been widely used by fisheries managers to evaluate stock supplementation programs, most are quite labor intensive as they require the handling and marking of individual fish. Recently, fisheries managers have become interested in methods for the mass marking of larval or juvenile fishes. For example, immersion marking with oxytetracycline hydrochloride (OTC) allows fish to be mass marked with minimal handling, low cost, and minimal labor. However, mark detection of OTC marked fish is quite a laborious process. Mark detection requires that fish be sacrificed, otoliths removed and mounted on microscope slides, and otoliths then viewed with a fluorescent microscope. Otolith processing and mounting alone requires approximately 30 min per fish. Consequently, there is strong interest among fish culturists and fishery managers for a method (or chemical) to mass mark fish where the mark can be detected easily, without sacrificing fish, and that can be completed in the field. Calcein may be such a chemical.

Calcein is a fluorochrome compound that binds to calcified structures such as fish otoliths, fin rays, scales, and bony structures around the opercular and jaw areas. Immersion marking of fish using calcein results in a bright green fluorescent mark when excited with blue light of about 500 nm. Calcein has been evaluated as a method of mass marking fish otoliths (Wilson et al. 1987; Beckman et al. 1990; Brooks et al. 1994; Bumguardner and King 1996) as well as fin rays, scales, and other calcified tissues (Alcobendas et al. 1991; Gelsleichter et al. 1997; Mohler 1997; Leips et al. 2001; Mohler et al. 2002). These studies indicated that immersion marking of fish using calcein resulted in a bright green fluorescent mark that was similar in width, intensity, and duration to the mark produced by OTC. Research conducted at the Services Northeast Fisheries Center (Lamar, PA) demonstrated that calcein not only marks otoliths, but also produces a brilliant green fluorescence in fin rays, scales, opercles, and jaw bones, and that these marks can be easily detected on live fish using a handheld fluorescent detector. Additional studies have also been conducted to demonstrate the longevity of marks, safety of calcein to fish, and whether any delayed fish health or adverse behavior effects were observed. Based on results of the above-described studies and the apparent utility of calcein as a means to mass-mark larval fish, the Service requested from FDA a compassionate INAD exemption for calcein. Because calcein is not approved for use in aquaculture or for use on other food animals

in the U.S., obtaining such an exemption (and authorization to stock or harvest treated fish) took nearly two years. However, FDA recently granted the Service an INAD exemption (INAD #10-987) including a slaughter authorization for calcein, which allows for the use of calcein as a marking agent according to guidelines established in the INAD study protocol.

SE-MARK[®], the trade name for calcein as a marking agent, is sponsored by Western Chemical Inc. (Ferndale, WA), and is available as a 1% active solution. Although SE-MARK[®] is a pH-balanced solution, the pH of the calcein working solution should be measured and adjusted so that it is close to 7. Under the study protocol for INAD #10-987, only fish weighing 2 gm or less may be marked with SE-MARK[®]. Marked fish of this size may be released at any time after treatment (i.e., no withdrawal period). The protocol allows for two static bath treatment regimens, a relatively slow regimen and a more rapid regimen. Use of the more rapid marking induction regimen should be preceded by salt solution treatment to facilitate osmotic uptake of calcein (note: each species should be tested for its salinity tolerance prior to marking!). To prepare the salt solution, use a non-iodized granular food grade salt and mix with hatchery water. For non-feeding salmonides, fish should be exposed to a 5% salt solution for 3.5 - 4 min. However, once fish develop scales and are being fed a hatchery diet, they should be exposed to a 1.5% salt solution for 3.5 - 4 min. The slow regimen allows treating fish for 1 - 6 h at a calcein concentration of 125 - 250 mg/L. The fast regimen allows treating fish for 1 - 7 min at a calcein concentration of 2,500 - 5,000 mg/L (2.5 - 5 g/L). If necessary, aeration should be provided to marking solutions via an air stone to maintain dissolved oxygen levels during marking episodes. In some cases, it may be desirable to mark a fish a second time. Managers should wait at least 2 d before making the second mark. They should also keep in mind that the longer the duration between marks, the greater the distinction there will be between marks. It is recommended that the concentration used to make the second mark should be one half the concentration used to make the first mark. It is also recommended that if scales or otoliths are to be collected and read at a later date or archived for historical purposes, they should be stored in grain or 100% denatured alcohol.

Mark detection procedures are completed using the commercially available SE-MARK[®] Fluorescent Detector. The detector is a handheld device that emits ultraviolet light at a wavelength of about 500 nm, and is available from Western Chemical Inc. for \$3,500 (U.S). Following treatment, the protocol recommends that the calcein mark should be read from a minimum of 15 fish, and that marks should be read a second time if fish are to be held on-station for more than 30 d. Managers should be aware that it may be necessary to lightly anesthetize fish (especially fish that are > 6 months old) for ease of reading marks. Quality of marks should be documented using an ordinal scale (i.e., 0, 1, 2, or 3; 0 being a poor or non-existent mark, 4 being an excellent mark). Make sure that the individual reading marks can see the color green! According to the protocol, you will need to document the elapsed time between when fish were marked and when marks were read and the condition of the fish when either marked or when marks were read.

Because of a lack of specific data on calcein, particularly with respect to the fate of calcein discharged into the environment, special disposal procedures have been implemented for calcein solution remaining in static baths following treatment. No discharge of calcein into receiving water will be permitted under INAD #10-987. All calcein solution remaining in static baths after treatment must be stored in secondary containers on station until disposal. If a gentle fresh-water spray is used to rinse excess calcein off a net or strainer full of fish before placing fish back into their culture units, spraying should take place over a tub to capture waste calcein. The captured calcein should then be combined with calcein waste from the static baths. At this time, the only disposal facility for which arrangements have been made to incinerate waste calcein solution is Emerald Services, Inc. (Tacoma, WA). Ship waste calcein solutions to Emerald Services in durable containers. Other disposal facilities and options are being investigated by the sponsor and the Service, and users of calcein under INAD #10-987 will be notified of additions to disposal options as they become available.

Calcein INAD #10-987 allows for the use of calcein on a production level, and will hopefully provide a useful tool for a broad variety of fisheries managers and stock enhancement programs. It is further hoped that based on data generated under INAD #10-987, we will be able to make an objective evaluation to determine if it is sensible to move forward and try to gain FDA approval for the use of calcein as a marking agent. If you are interested in using SE-MARK[®] under INAD #10-987, please contact Dave Erdahl (406-587-9265 ext. 125, dave_erdahl@fws.gov) or Bonnie Johnson (406-587-9265 ext. 136, bonnie_johnson@fws.gov). No fee will be charged to Service facilities or offices that wish to use calcein under this INAD. However, the standard fee will be charged to non-FWS users to offset costs associated with administering the INAD and reporting to FDA.

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