Attachment 3:

USFWS Technical Information Leaflet LM -02-01 : Analysis of Atlantic Salmon Tissues for Residual Calcein Fluorescence after Osmotic Induction of a Calcein Mark Using SE-MARK™

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Technical Information Leaflet No. LM-02-01

Analysis of Atlantic salmon tissues for residual calcein fluorescence after osmotic induction of a calcein mark using SE-MARKTM.

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Analysis of Atlantic salmon tissues for residual calcein fluorescence after osmotic induction of a calcein mark using SE-MARK™.

Background and Justification:

Fisheries restoration and recovery plans often rely on some type of fish marking to evaluate progress toward achieving management goals. Although there are currently numerous types of tagging and marking options available to fishery managers, each has it's own limitations. Therefore the search for additional tagging and marking technology continues. The fluorochrome compound commonly known as calcein has shown promise to provide an additional marking tool having capabilities of providing fisheries evaluations not possible or practical to perform with previous marking techniques. Calcein has been evaluated in laboratory experiments as a method of mass-marking fish otoliths as well as fin rays, scales, and other calcified tissues. It has also recently been shown to be a more sensitive and inclusive method for visualizing skeletal structures in fish as compared with other bone markers such as alcian blue and alizarin red (Du et al. 2001). In order to fully evaluate the usefulness of this emerging technology, an Investigational New Animal Drug (INAD) exemption must be obtained from the U.S. Food and Drug Administration (FDA) so that fishery managers and other investigators can legally mark and release fish for testing purposes. Obviously, it is important to understand the effects which any chemical or drug has on the target organism. It is equally important to determine potential drug residue effects relating to human food safety if the target animal is a potential food item. Part of this understanding involves gaining knowledge of drug residue levels in various fish tissues following treatment. In this study we evaluated the presence of residual calcein in blood, dorsal muscle, liver, kidney, and spleen from yearling Atlantic salmon following immersion in calcein.

Methods

The study took place at the Northeast Fishery Center (NEFC), Lamar, PA in April and May, 2002. Approximately 40 yearling Connecticut River strain Atlantic salmon (mean weight = 109 ; mean length = 94mm) were treated with calcein to induce a calcein mark on fin rays, scales, and other calcified tissues. Fish were batch-marked by immersion in 1.5% NaCI solution for 3.5 minutes followed immediately by immersion in a 0.5% calcein solution for 3.5 minutes. After marking procedures were completed, fish were placed into a culture tank supplied with flowthrough freshwater and cared for in a manner similar to other captive fish. A group of nonmarked control fish from the reference population was used for comparison of calcein residues in tissues. Calcein used in this study (trade name SE-MARK™) was supplied by Western Chemical, Inc., and was considered to be representative of the calcein that will be manufactured by Western Chemical for use under INAD exemption.

Two hours post-treatment, all fish were observed using a calcein detection device (patent pending) to determine the efficacy of the marking procedure. At the same time, 4 fish were randomly collected from the treated population for tissue sampling. Fish were euthanized by over-exposure to a Eugenol/alcohol immersion-bath immediately prior to tissue sampling. The caudal fin was severed and blood sampled via a capillary tube. Dorsal muscle, kidney, liver, and spleen samples were collected using clean dissecting instruments each time a sample was obtained. Tissue samples were placed on microscope slides and prepared as squash smears (Post

1987). Squash smears were examined via fluorescence microscopy for the presence of calcein and digital photos were taken to document results. Transmitted white light was bled onto the slide to highlight non-fluorescing structures in some micrographs. Fluorescence was subjectively scored on a graded scale as brilliant = 3, medium = 2, dim but detectable = 1, or none detectable = O. Other pertinent characteristics were also described. Subsequent samples were collected and evaluated at 24, 48, and 72 hours post-treatment and again at 10 and 21 days post-treatment.

In order to estimate the lowest concentration of SE-MARK™ calcein which could be visually detected using fluorescence microscopy, serial dilutions of the compound were prepared. Concentrations ranged from 10 to 1 x 10^{-5} ppm and were prepared from a 100 ppm stock solution using a micro-pipette. The stock solution was made by weighing out lOmg of SEMARK™ calcein on an analog analytical balance (Ainsworth Corporation, Denver, CO.) and mixing with 100 ml of hatchery water using a magnetic stirring device. The pH of the stock solution was titrated to 7.5 with a few drops of NaOH solution. A twentY-ill drop of each dilution sample was placed on 2 clean microscope slides; one had a cover slip applied and one did not. Each slide was viewed under fluorescence microscopy using the same equipment and settings used for viewing the previously mentioned salmon tissue samples. Visual fluorescence was then scored as present or absent for all dilutions.

Results

At two hours post-treatment, all fish were found to be brilliantly labeled externally with the fluorochrome SE-MARK™ fluorescing in scales and fin rays. No mortalities occurred throughout the 21-day study period. The 2-hour samples showed that all selected tissues exhibited some degree of green fluorescence characteristic of calcein. Observations from individual tissues are described as follows:

Blood.- Although blood cells from calcein-treated fish did not fluoresce, plasma exhibited a green hue 2 hours post-treatment and was given a score of 2 (medium fluorescence) (Figure 1). However, blood plasma was found to be devoid of any green hue or fluorescence at the lO-day post-treatment sample period and given a score of 0 (no detectable fluorescence) (Table 1). Blood samples from control fish showed no green hue in plasma.

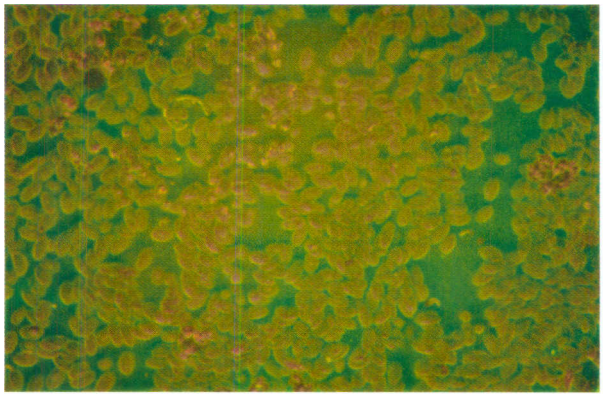

Figure 1. Blood smear of Atlantic salmon 24h post-immersion in SE-MARK™ calcein solution showing fluorescence of plasma but not of blood cells (400X)

Kidney.- Cells lining the excretory tubules of the kidney in SE-MARK™ treated fish exhibited brilliant fluorescence compared to non-marked controls. These structures exhibited the greatest fluorescence of all tissues evaluated, likely due to the presence of calcium salt concretions (Figure 2). Therefore the level of fluorescence of the excretory tubules was given a score of 3 (brilliant) and remained unchanged throughout the 21-day sampling period (Table 1). Kidney samples from control fish showed no fluorescence.

Table 1.- Levels of calcein fluorescence observed in tissues of yearling Atlantic salmon at various time periods post-treatment with SE-MARK ™. Values were subjectively scored on a graded scale as follows: 3=brilliant fluorescence, 2=medium fluorescence, l=dim but detectable fluorescence, and O=no detectable fluorescence.

Tissue type:	Time post-treatment:					
	2 hours	24 hours	48 hours	72 hours	10 days	21 days
muscle	1	1	1	1	a	a
blood	2	2	2	1	a	a
kidney	3	3	3	3	3	3
Ii ver	2	2	2	2	1	1
spleen	2	2	2	2	2	1

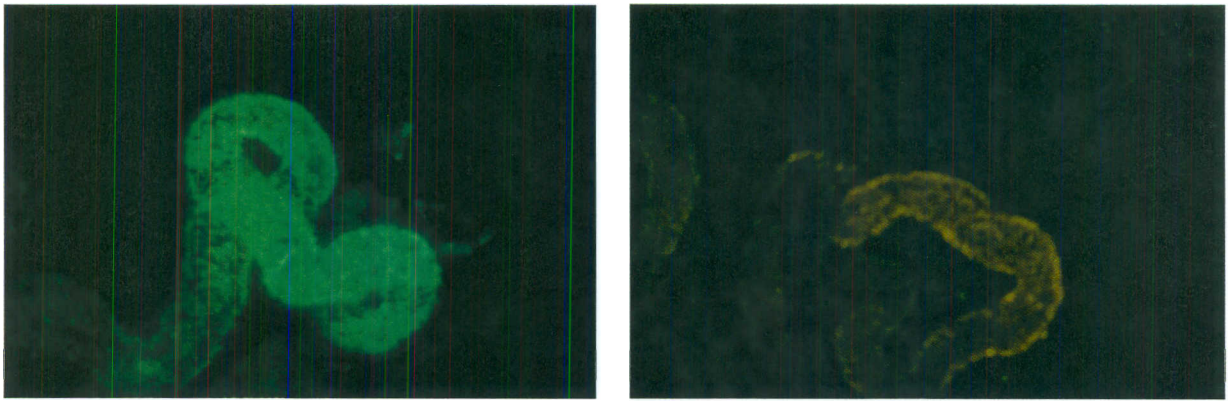

Figure 2.- Excretory tubules of Atlantic salmon (l) at 24h post-immersion in SE-MARK™ calcein solution; (r) non-marked control tubules *(200K)*

Muscle.- Detectable levels of fluorescence resulting from ca1cein treatment were found in muscle tissue at 2,24,48, and 72h post-treatment and were given a fluorescence score of 1 (dim, but detectable) at those sample periods (Figure 3). Muscle tissue from samples collected at 10 and 21 days post-treatment was found to be devoid of fluorescence and scored as a (Table 1). Muscle samples from control fish showed no fluorescence.

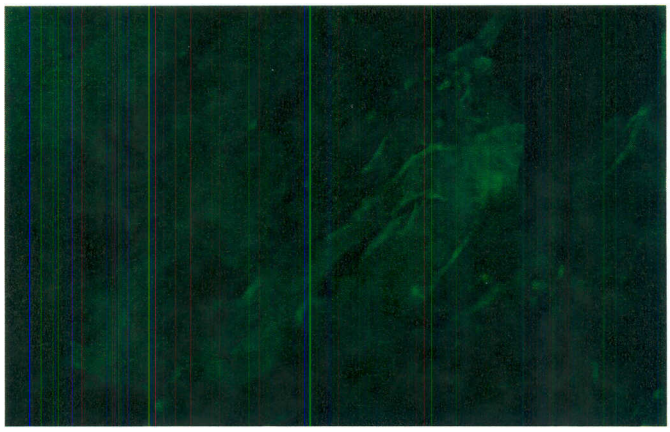

Figure 3.- Muscle tissue of Atlantic salmon showing some fluorescence at 2h post-immersion in SE-MARK™ ca1cein solution (lOaX)

Spleen.- Spleenic tissues from ca1cein-treated fish exhibited detectable levels of fluorescence (score = 2) in samples collected from 2 hours to 10 days post-treatment (Figure 4). However, only dim, but detectable fluorescence (score=l) was observed in the last sample period at 21d post-treatment even when maximum incident light power was applied to the microscope fluorcluster (Table 1). Splenic tissues from control samples showed no fluorescence.

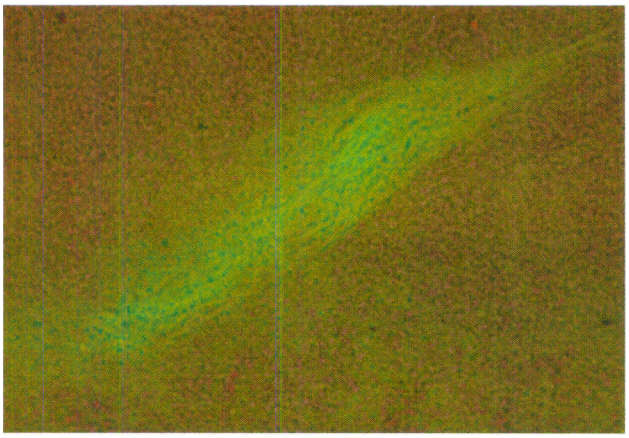

Figure 4.- Spleen squash of Atlantic salmon 24h post-immersion in SE-MARK™ ca1cein solution showing some fluorescence in connective tissue and adjacent cells (lOaX)

Liver.- Liver tissues from calcein-treated fish exhibited patterns and levels of fluorescence similar to that observed in spleen tissues. Samples collected at 2 hours post-treatment showed medium fluorescence (score=2), and levels of fluorescence remained unchanged through the 72hour sample period (Figure 5). At the lO-day sample period calcein fluorescence decreased to dim, but detectable levels (score=1). At the final 21d post-immersion sample period, liver tissues exhibited only traces of fluorescence when full incident light power was applied to the fluorocluster in the microscope (Table 1). Liver tissues from control samples showed no fluorescence.

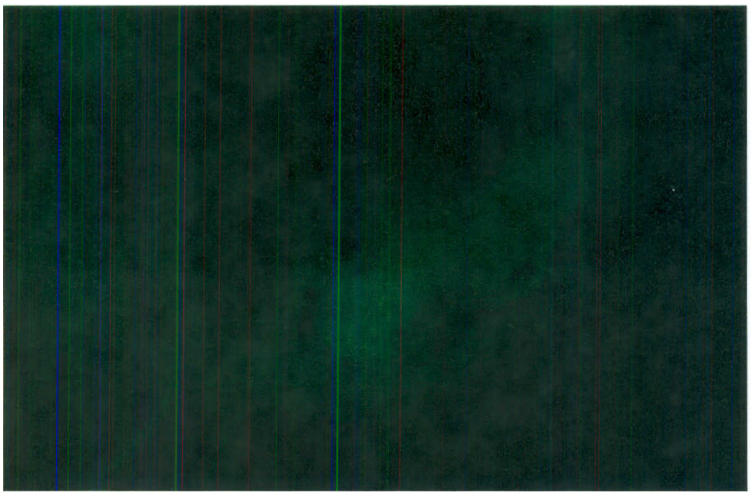

Figure 5.- Liver squash from Atlantic salmon at 24h post-immersion in SE-MARK™ calcein solution showing dim fluorescence in connective tissues and other liver tissue structures (100X).

Determination of visual fluorescence level using serial dilutions.- With the cover slip applied, visual fluorescence was easily detected at concentrations ranging from 10 to 10^{-2} ppm and barely detectable at 10-3 ppm level. However, in samples without the cover slip, fluorescence was easily detected at the 10-3 ppm level. At the lowest levels examined (10^{-4} and 10^{-5} ppm) visible fluorescence was not detectable in samples with or without the cover slip (Table 2).

Table 2.- Determination of lowest level of visually-detectable fluorescence in serial dilutions of SE-MARK™ calcein at concentrations ranging from 10 to 10-^{5} ppm.

Concentration level (ppm)		Visual fluorescence detection: With cover slip	Without cover slip
1 x 10^{1}	(10.0)	+	+
1 x 10°	(1.0)	+	+
1 X 10-^{1}	(0.1)	+	+
1 x 10-^{2}	(0.01)	+	+
1 X 10-^{3}	(0.001)	–	+
1 x 10-^{4}	(0.0001)		
1 X 10-^{5}	(0.00001)		

Discussion/Conclusions

Following immersion in a 0.5% SE-MARK™ calcein solution using osmotic induction procedures (pre-treatment in a 1.5% NaCI solution for 3.5 minutes), all internal tissues examined appeared to contain calcein residues as evidenced by microscopically-visual fluorescence as compared to non-exposed controls. Based on these preliminary observations, it is likely that other internal organs are also initially exposed to this compound and will bind calcein in proportion to the levels of calcium present in those tissues. In tissues where calcium salts or other calcified structures are permanently present, such as excretory tubules of the kidney, calcein residues will likely persist long-term.

The lowest level of visually-detectable fluorescence for SE-MARK™ calcein was found to be 10-^{3} ppm in the sample viewed without a cover slip and 10-^{2} ppm in the sample with the cover slip. Application of a cover slip to the liquid sample caused the sample droplet to be spread out in an even, thin layer over the entire surface area of the cover slip while the droplet viewed without the cover slip retained a greater thickness due to the inherent surface water tension in the droplet. In describing the use of mounting media in color photomicrography, DeIly (1988) states that the degree of yellowing depends upon the thickness of the mounting medium as well as it's age. Therefore in the current study, when the incident light penetrated a greater thickness of sample (i.e., the sample without a cover slip) the resulting fluorescence detection was enhanced due to the increased sample thickness as compared to the sample with the cover slip in place.

Perhaps most important with respect to a potential new animal drug approval for SE-MARK™ calcein in the current study were the observations that blood and muscle tissue were devoid of visually-detectable fluorescence caused by calcein residues at the lOd post-treatment sample period and beyond. Likewise, this finding suggests that in the yearling salmon tested, muscle and blood tissue had calcein residue levels between 10-^{2} and lO'3$_{ppm}$ during the same sample period post-exposure when the above-mentioned visual detection limits are applied. In describing the composition of a 24-g coho salmon, Smith (1982) reported that muscle tissue comprises about 56% of the total wet weight. If we apply this estimate to the lO-g wet weight of

our study fish we calculate that there are 5.6g of muscle tissue. Application of lowest level of visually-detectable calcein found in serial dilutions (without cover slip: $1O^{-3}$ppm = O.OOlppm = 1ppb = 1 x 10-9;) to an individual fish used in our study, we find that one individual contains less than 5.6 x $1O^{-9}$g of calcein residue in muscle tissues at this stage of development (Formula 1.1). This residue level estimation can then be applied by extrapolation to a salmon which could possibly enter the human food chain. At a total length of about 20 cm (8 in), an Atlantic salmon could possibly be mistaken for a legal-sized trout by an angler. A salmon at this size would be in it's second year of life and weigh about 83g (Gaston 1988). Assuming the salmon still contains the original 5.6 x 10^{-9} g of calcein in muscle tissue, but now has increased in muscle weight to 46.5g, we then calculate the level of residue at less than 1.2 x lO^{-lO}g of calcein per gram of muscle tissue or less than 1.2 parts per ten-billion (Formula 1.2). Applying the lowest level of visually-detectable calcein found in serial dilutions with the cover slip (10-zppm) to the same calculations would result in the estimation of less than 1.2 x $1O^{-9}$g of calcein per gram of muscle tissue in the 8-in fish.

(1.1) 5.6g(1 x 10^{-9}) = 5.6 x 10^{-9} g of calcein residue per fish in muscle tissue of a 10-g salmon

(1.2) 5.6 x 10^{-9} *g+46.5g* = 1.2 x 10^{-10} gof calcein residue per gram of muscle in an 83-g salmon

In the current study, tissue absorption of calcein was likely enhanced via creation of a high osmotic potential between the fish and their external environment through the use of a strong salt bath prior to exposure to SE-MARK™ solution (osmotic induction procedure). Lending support to this theory is the work of Conte (1969) which states that the marine environment causes an obligatory water loss from body fluids (through the skin, gills, and kidney to a minor extent) owing to the differential in osmotic concentrations. Using this as a model of the "water budget" in our fish during calcein-marking, we can predict that the fish will lose a certain amount of water to their environment when placed in the 3.5-min salt bath. Subsequently, when the fish are abruptly moved into a calcein solution, the resulting osmotic difference results in rapid uptake of the calcein solution as water is replaced via osmosis through the skin and gills.
Calcein binds calcium and other metal ions by chelation (Wallach et al. 1959; Hefley and Jaselskis 1974), therefore once it is introduced to the internal fluids of the fish, it can bind with and label calcified tissues.

Results of the current study lay a foundation for future calcein SE-MARK™ tissue residue studies with other species and life stages of aquatic organisms. Furthermore, these results can be used as the best currently-available information concerning calcein tissue residue levels, and also help establish important withdrawal time requirements for fish treated with calcein (SE-MARK™) before they can be allowed to enter the human food chain.

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