AQUACULTURE DRUGS: DETERMINATIVE METHOD FOR THE AQUI-S® MARKER RESIDUE IN FILLET TISSUE[2]

Project ***Termination Report*** for the Period
January 1, 2006 to August 31, 2008

NCRAC FUNDING: $129,936 (January 1, 2006 to December 31, 2006)

PARTICIPANTS:

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REASON FOR TERMINATION

All work was completed. A final report describing the validation of a proposed determinative method for the AQUI-S® marker residue was submitted to U.S. Food and Drug Administration's Center for Veterinary Medicine (CVM). However because of a ruling identifying isoeugenol (active ingredient of AQUI-S®) as a carcinogen, Objective 4 could not be completed.

PROJECT OBJECTIVES

(1) Interact with the CVM to determine the requirements and procedures to develop and validate a determinative analytical method for the AQUI-S® marker residue in all cool and warm water species of fin fish.

(2) Develop and validate a determinative analytical method for the AQUI-S® marker residue in all cool and warm water species of fin fish according to CVM guidelines for method development under Good Laboratory Practices.

(3) Write the final study report and submit the report to an Investigational New Animal Drug (INAD) number established by CVM for AQUI-S®.

(4) Gain acceptance from CVM for the determinative analytical method for the AQUI-S® marker residue that will help support the approval of AQUI-S® for short-exposure handling for all cool and warm water species of fin fish.

[2]NCRAC has funded seven Aquaculture Drugs projects. A termination report for the first project is contained in the 1997-98 Annual Progress Report; a termination report for the second project is contained in the 1996-97 Annual Progress Report, a termination report for the third project is contained in the 2001-02 Annual Progress Report, a termination report for the fourth project is contained in the 2006-07 Annual Progress Report, and a termination report for the sixth project is contained elsewhere in this report. A fifth project, which provided $60,000 for a portion of the funds required to purchase sufficient radiolabeled AQUI-S® for use in a total residue depletion study in rainbow trout, is also reported on under the progress report for the National Coordinator for Aquaculture New Animal Drug Applications (NADAs) elsewhere in this report. This termination report is for the seventh Aquaculture Drugs project which is being undertaken by Jeffrey R. Meinertz. It was a 1-year project that began January 1, 2006.

PRINCIPAL ACCOMPLISHMENTS

A study protocol was developed and submitted to CVM. The protocol was returned with review comments which were used to revise the protocol. The chemical purity of the test chemical was verified with high performance liquid chromatography techniques. The instrument (high performance liquid chromatography system) detection and quantitation limits were determined for isoeugenol analytical standards prepared with 90:10 methanol:water. The loss of isoeugenol from solutions prepared with 90:10 methanol:water was evaluated periodically through a 21-day storage period.

Fillet tissue from unexposed fish was acquired from the following species: brown trout (*Salmo trutta*), channel catfish (*Ictalurus punctatus*), fall Chinook salmon (*Oncorhynchus tshawytscha*), hybrid striped bass (*Morone saxatilis* × *M. chrysops*), lake trout (*Salvelinus namaycush*), largemouth bass (*Micropterus salmoides*), northern pike (*Esox lucius*), walleye (*Sander vitreus*), and yellow perch (*Perca flavescens*). The fillet tissue from each species was homogenized with dry ice in preparation for impending studies requiring homogenized control fillet tissue. Homogenized control fillet tissue from lake trout was processed with the proposed determinative method for an evaluation of chromatographic interference that would interfere with the determination of isoeugenol concentrations in lake trout fillet tissue.

Brown trout, channel catfish, hybrid striped bass, lake trout, largemouth bass, northern pike, walleye, and yellow perch were exposed to AQUI-S® (a separate exposure for each species) for the purpose of generating biologically-incurred isoeugenol residues in the fillet tissue. Generation of fillet tissue with biologically-incurred isoeugenol was necessary for the evaluating method precision with fillet tissue containing biologically-incurred isoeugenol residues and for evaluating isoeugenol stability in fillet tissue stored at <-70°C (- 94°F).

The precision of the proposed determinative method was evaluated with brown trout, channel catfish, hybrid striped bass, lake trout, largemouth bass, northern pike, walleye, and yellow perch fillet tissue containing biologically-incurred isoeugenol. The loss of isoeugenol from fillet tissue containing biologically-incurred isoeugenol and stored for about 1 month at <-70°C (-94°F) was evaluated with brown trout, channel catfish, hybrid striped bass, and lake trout fillet tissue.

Samples of homogenized control fillet tissue were processed with the proposed determinative method for an evaluation of fillet constituents that would interfere with the determination of isoeugenol concentrations. Control tissue from the following species was processed: brown trout, channel catfish, fall Chinook salmon, hybrid striped bass, largemouth bass, northern pike, walleye, and yellow perch.

The method detection and quantitation limits were determined with isoeugenol-fortified fillet tissue as were method accuracy and within-day precision from the following species: brown trout, channel catfish, fall Chinook salmon, hybrid striped bass, lake trout, largemouth bass, northern pike, walleye, and yellow perch.

The method accuracy and within-day precision were determined with isoeugenol- fortified fillet tissue from the following species: brown trout, channel catfish, Fall Chinook salmon, hybrid striped bass, lake trout, largemouth bass, northern pike, walleye, and yellow perch.

The method day-to-day precision was determined with isoeugenol fortified fillet tissue from channel catfish.

The loss of isoeugenol from extracts generated with fortified fillet tissue was determined after 1, 7, and 14 days of storage. Control tissue from the following species was fortified with isoeugenol: brown trout, channel catfish, fall Chinook salmon, hybrid striped bass, lake trout, largemouth bass, northern pike, walleye, and yellow perch.

The loss of biologically-incurred isoeugenol from fillet tissue from the following species and stored at <-70°C (-94°F) was determined after 1, 2, 3, 4, 5, and 6 months: largemouth bass, northern pike, walleye, and yellow perch.

The loss of biologically-incurred isoeugenol from fillet tissue stored at <-70°C (-94°F) was determined after 2, 3, 4, 5, and 6 months of storage. Fillet tissue from the following species was assessed: brown trout, channel catfish, hybrid striped bass, and lake trout.

The loss of biologically-incurred isoeugenol from fillet tissue was determined after subjecting fillet tissue to three freeze/thaw cycles. Fillet tissue from the following species was assessed: brown trout, channel catfish, hybrid striped bass, lake trout, largemouth bass, northern pike, walleye, and yellow perch. In summary, the method was developed to use relatively common procedures and equipment. The procedures include extracting isoeugenol from tissue with acetonitrile, evaporating the acetonitrile from the extract with rotary evaporation techniques, changing the polarity of the extract by adding water, concentrating the isoeugenol with solid phase extraction procedures, and determining concentrations with high pressure liquid chromatography.

The method is robust, i.e., the method will produce accurate and precise results with fillet tissue from the following fish species: brown trout, channel catfish, Chinook salmon, hybrid striped bass, lake trout, largemouth bass, northern pike, walleye, and yellow perch.

The method is accurate, i.e., the percentage of isoeugenol recovered from samples fortified with isoeugenol at nominal concentrations of 1, 50, and 100 µg/g for all species was always >80.3% and <96.5%.

The method is repeatable, i.e., the within- day precision for samples fortified at nominal concentrations of 1, 50, and 100 µg/g for all species was ≤8.5% relative standard deviation (RSD). The day-to-day precision with fillet tissue fortified at a nominal isoeugenol concentration of 1, 50, and 100 µg/g is ≤3.0% RSD. The method precision with tissue from all species containing biologically-incurred isoeugenol was ≤8.1% RSD with the exception of fall Chinook salmon (live fish were not available).

The method is specific, i.e., there are no chromatographic interferences in extracts from control fillet tissue from brown trout, channel catfish, hybrid striped bass, walleye, and yellow perch and only minimal interferences (<0.11 µg/g, isoeugenol equivalent concentration) in the extracts from control fillet tissue from lake trout, largemouth bass, and northern pike. More notable interference was found in the fillet tissue extracts from fall Chinook salmon (0.20 to 0.52 µg/g, isoeugenol equivalent concentration).

The method is sensitive, i.e., the method detection limits for all species, except for fall Chinook salmon, range from 0.004 to 0.014 µg/g and the quantitation limits range from 0.012 to 0.048 µg/g. The method detection limit for fall Chinook salmon is 0.99 µg/g and the method quantitation limit is 3.3 µg/g. Isoeugenol in the various matrices was moderately stable. Loss of isoeugenol was insignificant in 90:10 methanol:water solutions with nominal isoeugenol

concentrations of 0.1 and 10 µg/mL stored for at least 14 days. Isoeugenol concentration changes are <10% in fillet tissue extracts from 6 of 9 species with nominal isoeugenol concentrations of 1, 50, and 100 µg/mL stored for 14 days. Isoeugenol concentration changes are <10% in fillet tissue from all species stored at <-70°C (-94°F) for 6 months. Isoeugenol concentration changes are <10% in fillet tissue from 6 of 8 species subjected to freeze/thaw cycles.

A comprehensive final report describing the study results was reviewed for accuracy and compliance with FDA regulations for good laboratory practices by the Upper Midwest Environmental Sciences Center (UMESC) Quality Assurance Officer. Because of the following statement issued in late April 2007, submission of the report to CVM for review and submission to INAD number 11- 475 for AQUI-S® was postponed:

"Isoeugenol (the active ingredient in AQUI-S®) has been under evaluation by the National Toxicology Program (NTP), an interagency program whose mission is to evaluate chemical agents for potential public health risks.

Recently, NTP was forced to delay the review of their nearly completed two- year toxicology studies on isoeugenol until February 2008 because of higher priorities. Although the study data have not been fully analyzed, the preliminary assessments of the data do not eliminate the possibility that isoeugenol residues in treated fish could pose a human health risk.

Because we need to be absolutely certain that there are no human food safety issues that would preclude the approval of AQUI-S®, the U.S. Fish & Wildlife Service (FWS) and the U.S. Geological Survey (USGS) and other participating partner groups have agreed to institute interim measures that will be effective until the NTP meeting in February 2008. Effective April 27, 2007, all ongoing and planned AQUI-S® research funded under the Association of Fish and Wildlife Agencies' Multi-State Conservation Grant, and allied work supported with federal base funds of FWS and USGS will be suspended until the completion of the NTP review. Additionally, FWS will temporarily suspend all field activities under their Investigational New Animal Drug exemption for AQUI-S® until the NTP review is complete.

Although the decision to temporarily suspend all publicly funded AQUI-S® research activities was not an easy decision to make, as responsible stewards of public funds it is the correct course of action. It should be noted that significant portions of the data necessary to address many of the original AQUI- S® goals and objectives of the Federal- State Aquaculture Drug Approval Partnership Project have already been generated. It is also important to note that USGS is constrained from further development of residue chemistry data until a tolerance value for the residues has been established by the Center for Veterinary Medicine. This work cannot be initiated until the results of the NTP studies are finalized. We look forward to the opportunity of continuing our collaborative AQUI-S® research efforts in February 2008."

Then in early March 2008, the following statement concerning the status of AQUI- S® was posted:

> "Isoeugenol (the active ingredient in AQUI-S®) has been under evaluation by the National Toxicology Program (NTP), an interagency program whose mission is to evaluate chemical agents for potential public health risks. Initial results reported from the NTP studies resulted in cessation of drug approval efforts for AQUI-S® by the federal partners on the Association of Fish and Wildlife Agencies (AFWA) Drug Approval Working Group (DAWG) on April 27, 2007. On February 28, 2008, the NTP peer review panel confirmed that there is clear evidence of isoeugenol carcinogenicity in male mouse livers; there was no or equivocal evidence of carcinogenicity for the female mouse and male and female rat. Finding clear evidence of carcinogenicity in the male mouse triggered the Delaney Clause, a 1958 amendment to the Food, Drugs, and Cosmetic Act (FDCA). The clause states that "the Secretary of the Food and Drug Administration shall not approve for use in food any chemical additive found to induce cancer in man, or, after tests, found to induce cancer in animals". The Center for Veterinary Medicine (CVM) recently stated that it was "very, very unlikely" that a zero-withdrawal period could be gained for isoeugenol based on the NTP interpretation of the results of the male mouse study and the application of the Delaney Clause to the FDCA."

A decision was made to submit to CVM a comprehensive final report that summarized the results of this work. The report was titled "Evaluation of a proposed determinative method for determining concentrations of isoeugenol in fillet tissue from cold, cool, and warm water fish species." The report was submitted for inclusion into INAD number 11-475. We did not request review of the report at this time. We did request that the report be forwarded to the Center for Food Safety and Applied Nutrition for their determination of whether or not the method described in the report could be used in their monitoring program.

IMPACTS

To support FDA approval of a new animal drug for fish, a series of toxicology and residue chemistry studies are conducted to demonstrate the safety of food products derived from treated fish. Mammalian toxicology studies determine if the drug is safe for humans to consume and the amount of drug residues that can be consumed daily for a lifetime without causing adverse effects (acceptable daily intake; ADI). Considering the amount of tissue consumed in a lifetime, the ADI is used to calculate a safe concentration for all of the drug's residues in the edible tissue.

Residue chemistry studies are conducted to assess drug residues in the edible fillet tissue from treated fish. First a total drug residue depletion study is conducted to identify all of the drug's residues in the edible fillet tissue and characterize the depletion of those residues from the fillet. Based on data from this study, a marker residue is selected. The marker residue is one compound or group of compounds that will represent all of the drug's residues in subsequent depletion studies.

After selection of a marker residue, analytical methods for the marker residue are developed and validated. Two methods are required, a determinative method (activities described in this report were conducted to fulfill requirements for a determinative method) and a confirmatory method. The determinative method quantifies concentrations of the marker residue in edible tissue. The confirmatory method confirms the results from the determinative method and provides irrefutable identification of the marker residue in the tissue.

After validating a determinative method, marker residue depletion studies are conducted. Data from these studies are used in conjunction with the safe concentration to determine a tolerance concentration for the marker residue, as well as a withdrawal time. The tolerance concentration is the concentration of the marker residue in the edible tissue that represents the safe concentration (the concentration of all drug residues that is considered to be safe). The withdrawal time is the time it takes for the fish to deplete all drug residues to the safe concentration.

The total residue depletion study for AQUI- S® was completed in 2005. Based on the results from the total residue depletion study, isoeugenol would most likely have been selected as the marker residue. However, because of the information described in the notifications previously presented, all FDA decisions concerning AQUI-S® were postponed, including the selection of a marker residue. Nonetheless, all work validating a determinative method for the probable marker residue was completed. With the completion of that work, we would have been poised to develop and validate a confirmatory method for the probable marker residue as well as conduct the AQUI-S® marker residue depletion studies. Because of the decision to stop all work concerning AQUI-S®, we cannot continue developing data for AQUI- S®.

RECOMMENDED FOLLOW-UP ACTIVITIES

If a decision is made in the future to pursue AQUI-S® as an anesthetic with a longer withdrawal time, the next steps toward an approval will be for FDA to calculate an ADI, calculate a safe concentration, accept data from the total residue depletion study, officially select isoeugenol as the marker residue, and review and accept data from the validation of the determinative method. Additionally, a confirmatory method will need to be developed and validated for the marker residue and at least three marker residue depletion studies will need to be conducted. Data from those studies would be submitted to the FDA for their review and acceptance.

PUBLICATIONS, MANUSCRIPTS, OR PAPERS PRESENTED

See the Appendix for a cumulative output for all NCRAC-funded Aquaculture Drugs activities.

SUPPORT

YEAR	NCRAC-USDA FUNDING	OTHER SUPPORT					TOTAL SUPPORT
		UNIVER-SITY	INDUSTRY	OTHER FEDERAL	OTHER	TOTAL	
2006	$129,936			$30,044[a]		$30,044	$159,980
TOTAL	$129,936			$30,044		$30,044	$159,980

[a]UMESC salary costs for a GS13 and GS11 (4 pay periods each) that were accrued during the 4th quarter of calendar year 2006.