DETERMINATIVE METHOD FOR THE AQUI-S® MARKER RESIDUE IN FILLET TISSUE

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Funding Request: $129,936

Duration: 1 year (January 1, 2006-December 31, 2006)

Objectives:

1. Interact with the U.S. Food and Drug Administration's Center for Veterinary Medicine (CVM) to determine the data required to validate a determinative method for the AQUI-S® marker residue in fillet tissue from all species of coolwater and warmwater finfish.

2. Validate a proposed determinative method for the AQUI-S® marker residue in fillet tissue from all species of coolwater and warmwater finfish following Good Laboratory Practices.

3. Submit to an AQUI-S® Investigational New Animal Drug exemption a final report describing validation of a proposed determinative method for the AQUI-S® marker residue in fillet tissue from all species of coolwater and warmwater finfish.

4. Gain from CVM approval of the proposed method to be used as the official determinative method for the AQUI-S® marker residue in fillet tissue from all species of coolwater and warmwater finfish.

Proposed Budget:

Institution	Principal Investigator	Objectives	Year 1
USGS, Upper Midwest Environmental Sciences Center	Jeffery R. Meinertz	1 - 4	$129,936
		Total	$129,936

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JUSTIFICATION

Currently, Finquel (MS-222) is the only fish anesthetic approved by the U.S. Food and Drug Administration (FDA). Use of this anesthetic is constrained by a 21-day withdrawal period. A critical need for use of an anesthetic with zero withdrawal time in U.S. public aquaculture and fishery management has been expressed. A zero withdrawal anesthetic would allow anesthetized fish to be handled and released immediately after conducting nearly all fish culture and management procedures including transport, spawning, marking, harvesting, and grading.

AQUI-S® is a fish anesthetic approved in Australia, Chile, the Faeroe Islands, Korea, and New Zealand and is being pursued for approval in the U.S. as a zero withdrawal anesthetic. AQUI-S® was chosen for development as a zero withdrawal anesthetic for the following reasons: (1) it is the only candidate zero withdrawal anesthetic under development; (2) immediate funding was recently made available to generate data necessary for its approval; (3) it has an active sponsor who is providing major funding for data generation; (4) product formulation for the chemical will most likely meet Good Manufacturing Practices requirements; (5) its effectiveness has been proven with various fish species; and (6) preliminary results have shown it to be safe to fish, humans, and the environment.

The timely approval of AQUI-S® as a zero withdrawal anesthetic for short exposure handling of all freshwater fish depends upon timely funding for the completion of eight technical sections (or data categories). Currently, funding has been secured from a variety of sources to complete all the known FDA Center for Veterinary Medicine (CVM) data requirements with the exception of portions of the Human Food Safety and Target Animal Safety data categories. Outstanding and unfunded work includes validation of a determinative method for the AQUI-S® marker residue in fillet tissue from coolwater and warmwater fish species.

To support the approval of a new animal drug for fish, a series of toxicology studies and residue chemistry studies are performed to demonstrate the safety of food products derived from the treated fish. Data from these studies are submitted to the human food safety data category. The toxicology studies are generally performed in mice or rats and in dogs as models of human toxicity in order to determine the biological, pharmacological, mutagenetic, carcinogenetic, and reproductive effects. The results from the toxicology studies are used to establish an acceptable daily intake (ADI) which represents an amount of drug that can be consumed over the lifetime of the individual without harmful effects. The ADI is then used to calculate the safe concentration for residues of the drug in the human diet. The importance of the safe concentration will become apparent in the following text.

The residue chemistry studies are performed to assess residues of the drug in fish fillet tissue. The following studies are required to assess the residue chemistry of a drug: (1) a study to characterize the depletion, distribution, and nature of all drug residues in fillet tissue from one fish species (total residue depletion study); (2) studies to validate an analytical method for determining concentrations of a drug's marker residue in fillet tissue from all fish species from all temperature groups (the determinative method); (3) studies to validate an analytical method for confirming the identity of a drug's marker residue in fillet tissue from all fish species from all temperature groups (the confirmatory method); and (4) studies to measure over time the depletion of the drug's marker residue from fillet tissue from fish species from all temperature groups (marker residue depletion study).

A marker residue is selected from the total residue depletion study. Drug residues that make up at least 10% of the total residues are characterized so that the marker residue can be identified and selected. The selected marker residue is the residue that persists for the longest time in the target tissue and must be a compound suitable for analysis.

After selection of a marker residue, a tolerance concentration for the marker residue is determined. The tolerance is the concentration of the marker residue in the edible fillet tissue at the time when the total residues deplete to the safe concentration (previously defined). After determining the tolerance for the marker residue, a determinative method for the marker residue can be developed and validated for routine monitoring of the marker residue. After the determinative method has been accepted by CVM, marker residue depletion studies are conducted to set a withdrawal time for fish exposed to the drug.

In the case of AQUI-S®, residue chemistry data gaps are prevalent. Results from toxicology studies conducted by the National Toxicology Program (NTP) are not yet available, a total residue depletion study in rainbow trout is not yet complete, a marker residue has not been selected, a tolerance concentration for the marker residue has not been established, determinative or confirmatory methods for the marker residue in any fish species have not been accepted, and marker residue depletion studies in any fish species have not been conducted. However, the data gaps will soon be filled with data from studies in progress or from studies planned with obligated funds. An unfunded data gap is the validation of a determinative method for the AQUI-S® marker residue in fillet tissue from all species of coolwater and warmwater fin fish

RELATED CURRENT AND PREVIOUS WORK

The total residue depletion study for AQUI-S® was conducted by the project leader at the USGS Upper Midwest Environmental Sciences Center (UMESC) in La Crosse, Wisconsin. Before the study was conducted two pilot studies were conducted by the project leader. The first study was designed to study to determine the exposure conditions that would be used during the total residue study. Results from this pilot study indicated that isoeugenol concentrations would be greatest in fillet tissue from rainbow trout exposed for 60 min in water with a temperature of 17°C (62.6°F) and an AQUI-S® concentration of about 34 mg/L (ppm).

The second pilot study conducted by the project leader was designed to determine optimal sample times for characterizing the depletion of drug residues during the conduct of the total residue depletion study. Results from this study indicated that rainbow trout exposed to AQUI-S® should be sampled within four hours after removing the fish from the exposure bath.

Data from each pilot study were presented to CVM during discussions to develop a protocol for the total residue depletion study. Exposure and sampling parameters were negotiated and incorporated into the protocol.

The laboratory phase of the total residue depletion study is complete. Final modifications to a draft of the final report are nearly complete. The final report will be submitted to CVM before December 31, 2005. Data from the total residue depletion study indicate that isoeugenol is the primary residue in fillet tissue from rainbow trout exposed to AQUI-S®. Therefore, isoeugenol will likely be selected as the marker residue for AQUI-S®.

The project leader is also currently conducting a study to validate a proposed determinative method for isoeugenol in fillet tissue from a coldwater fish species, rainbow trout. The laboratory phase of the study is projected to be completed by January 31, 2006. A final report from this work will be submitted to CVM. The data provided to CVM will show that determinative method criteria will be fulfilled when the method is applied to fillet tissue from a coldwater fish species. Additional data will be required to show that the method is suitable for use with fillet tissue from coolwater and warmwater fish species before the method will be approved as the determinative method for the AQUI-S® marker residue.

ANTICIPATED BENEFITS

Acceptance by CVM of the proposed determinative method for the AQUI-S® marker residue will allow the required marker residue depletion studies to be conducted (by the UMESC). After CVM accepts data from marker residue depletion studies and accepts a proposed confirmatory method (developed by the UMESC), the Human Food Safety data category for AQUI-S® will be complete.

OBJECTIVES

1. Interact with the CVM to determine the data required to validate a determinative method for the AQUI-S® marker residue in fillet tissue from all species of coolwater and warmwater finfish.

2. Validate a proposed determinative method for the AQUI-S® marker residue in fillet tissue from all species of coolwater and warmwater finfish following Good Laboratory Practices.

3. Submit to an AQUI-S® Investigational New Animal Drug exemption a final report describing validation of a proposed determinative method for the AQUI-S® marker residue in fillet tissue from all species of coolwater and warmwater finfish.

4. Gain from CVM approval of the proposed method to be used as the official determinative method for the AQUI-S® marker residue in fillet tissue from all species of coolwater and warmwater finfish.

PROCEDURES

Interact with the CVM to determine the data required (Objective 1)

The project leader will contact CVM and discuss the study design that would ultimately be used for this study. The project leader has previous experience working with FDA in designing study protocols for validating marker residue determinative methods. The protocol will be written with guidance described in CVM guideline 3, "General principles for evaluating the safety of compounds used in food-producing animals", section IV, "Guideline for approval of a method of analysis for residues" and based on previously approved protocols for other drugs submitted to CVM.

Validate a proposed determinative method (Objective 2)

The procedures that follow are written in a format that will be used to construct the protocol that will be submitted to the CVM. Accordingly, there is some redundancy with other sections of this proposal.

Title
Evaluation of a proposed determinative method to determine concentrations of the AQUI-S® marker residue in fillet tissue from coolwater and warmwater fish species

Testing Facility:
Upper Midwest Environmental Sciences Center (UMESC)
Biological Resources Division
U.S. Geological Survey
2630 Fanta Reed Road
La Crosse, Wisconsin 54603

UMESC Study Number: CAP-06-AQUIS-06
Proposed Experimental Start Date: January, 2006
Proposed Experimental End Date: December, 2006

Reviewed and Approved by:

William H. Gingerich UMESC Branch Leader Chemistry and Physiology	Date	Christine M. Custer UMESC Chairperson Animal Care and Use Committee	Date
Mark P. Gaikowski UMESC Branch Statistician Chemistry and Physiology	Date	David M. Kennedy UMESC Quality Assurance Officer	Date
Linda E. Leake UMESC Acting Center Director	Date		

Approved by:

_____________________________ __________
Jeffery R. Meinertz Date
Project Leader

1. Preface

The work described in this protocol will be conducted as a regulated study. The data will be submitted to the Center for Veterinary Medicine (CVM) to support acceptance of the described method as a determinative method. The work described in this protocol assumes that isoeugenol will be selected as the marker residue for AQUI-S®. A tolerance concentration for the AQUI-S® marker residue has not been established, but is projected to be >1 *u*g/g and <100 *u*g/g.

2. Introduction

There is a critical need in U.S. public aquaculture and fisheries management for an anesthetic with zero withdrawal time. Such an anesthetic would have applications for nearly all fish species. For field studies, including population estimates and age and growth studies conducted by federal and state conservation agencies, fish could be handled with minimal stress and released immediately. A zero withdrawal time anesthetic would increase the effectiveness of stocking programs by decreasing stress related mortalities that occur during and after transport of fish. The availability of an anesthetic with no withdrawal time would also allow human consumption of spawned fish, such as Pacific salmon, immediately after exposure. Currently, Finquel (tricane methanesulphonate or MS-222) is the only fish anesthetic approved by the U.S. Food and Drug Administration (FDA). Use of this anesthetic to collect field data, spawn fish, or sedate fish during transport is constrained by a 21-day withdrawal period.

The approval of AQUI-S® as a fish anesthetic with a zero withdrawal time is being pursued in the U.S. As part of the approval process, a marker residue for AQUI-S® must be selected from AQUI-S® residues extracted from edible fillet tissue from exposed fish. After selection of the marker residue, a determinative method for the marker residue must be developed and validated in a variety of fish species from the colwater, coolwater, and warmwater temperature groups according to FDA guidelines. Thereafter, AQUI-S® marker residue depletion studies can be conducted.

Recently, a total residue depletion study was conducted at the Upper Midwest Environmental Sciences Center (UMESC). Data from the study indicated that isoeugenol was the primary residue in extracts from rainbow trout (*Oncorhynchus mykiss*) skin-on fillet tissue after exposure. Since isoeugenol was the primary residue in rainbow trout fillet tissue, we will assume isoeugenol will be selected as the marker residue for AQUI-S®.

Assuming isoeugenol is selected as the marker residue, a tolerance concentration for isoeugenol will eventually be declared by the Center for Veterinary Medicine (CVM) after all mammalian toxicology tests are completed and evaluated. Teratogenicity and carcinogenicity tests with isoeugenol have been conducted by the National Toxicology Program (NTP). Data from those tests are undergoing review by NTP. Based on current information, a tolerance concentration for the AQUI-S® marker residue will not be established until 2006. Despite the lack of a tolerance concentration for the AQUI-S® marker residue, we will conduct work that will address data requirements for a proposed AQUI-S® marker residue determinative method.

A method was developed at the UMESC to determine isoeugenol concentrations in fillet tissue from rainbow trout, a coldwater fish species. The method must be evaluated with fillet tissue from coolwater and warmwater fish species before it can be considered as the determinative method for the AQUI-S® marker residue. This study will evaluate the method with guidance described in CVM guideline 3, "General principles for evaluating the safety of compounds used in food-producing animals," section IV, "Guideline for approval of a method of analysis for residues." The isoeugenol concentrations chosen for investigation include a relatively wide range of concentrations and were chosen by incorporating current knowledge of isoeugenol concentrations in rainbow trout fillet tissue after exposing fish to AQUI-S®.

3. Objectives

The following objectives were established to support a determinative method for isoeugenol in edible fillet tissue from coolwater and warmwater fish species: (1) evaluate method interferences from naturally endogenous compounds extracted from control fillet tissue from coolwater and warmwater fish species; (2) evaluate method systematic error by assessing percent recovery with fillet tissue from coolwater and warmwater fish species fortified with isoeugenol at 1, 50, and 100 *u*g/g; (3) evaluate method repeatability by assessing within-day and day-to-day precision with fillet tissue from coolwater and warmwater fish species fortified with isoeugenol at 1, 50, and 100 *u*g/g; (4) evaluate method repeatability with biologically

incurred isoeugenol residues in fillet tissue from coolwater and warmwater fish species; (5) evaluate method detection and quantitation limits with fillet tissue from coolwater and warmwater fish species; (6) evaluate the stability of isoeugenol in fillet tissue extracts of fillet tissue from coolwater and warmwater fish species; and (7) evaluate the stability of isoeugenol in fillet tissue from coolwater and warmwater fish species containing biologically incurred isoeugenol residues.

4. Methods

4.1. Test article

The chemical structure of isoeugenol (2-methoxy-4-propenyl phenol; CAS # 97-54-1) is shown in Figure 1. Food grade isoeugenol (12 % cis isomer and 88% trans isomer, molecular weight, 164.2) will be obtained from AQUI-S New Zealand LTD (Lower Hutt, New Zealand). The subsample of the chemical will be archived according to procedures described in UMESC SOP GEN 011, “Archiving of regulated test chemicals.” The study director will ensure that a chemical log book is maintained for the chemical (UMESC SOP GEN 012, “Receipt, identification, storage, handling, checkout, and documentation of use of test chemicals”). Purity of isoeugenol will be determined or verified with a reversed-phase high pressure liquid chromatography (LC) system equipped with a diode array detector and mass spectrometer. Purity will be assessed by comparing chromatograms of an isoeugenol solution with chromatograms of the solvent used to prepare the isoeugenol solution. The area of peaks in chromatograms of the solvent will be subtracted from the area of peaks with similar retention times in chromatograms of the isoeugenol solution. All peak areas greater than 0 will be summed (total peak area). The peak area representing isoeugenol will be summed, multiplied by 100, and divided by the total peak area to calculate the percent purity.

4.2. Experimental animals

A variety of coolwater fish species will be difficult to acquire, however, an attempt will be made to acquire three coolwater fish species. Yellow perch (*Perca flavescens*) will be acquired from the UMESC fish culture facility. Additional coolwater species acquired from other fish culture facilities may include muskellunge (*Esox masquinongy*), northern pike (*Esox lucius*) and walleye (*Stizostedion vitreum*). Warmwater species, channel catfish (*Ictalurus punctatus*), hybrid striped bass (*Morone saxatilis* x *M. chrysops*), and largemouth bass (*Micropterus salmoides*) will be acquired from the UMESC fish culture facility.

cis isomer

trans isomer

Figure 1. Chemical structures of the isomers of isoeugenol.

Fish cultured at the UMESC will be reared in flowing well water with water characteristics similar to those listed in Table 1. Fish care and maintenance procedures during rearing will follow UMESC animal care and maintenance guidelines (UMESC SOP GEN 132, “Care, maintenance, and disposal of aquatic vertebrates”). Fish will be designated as acceptable for admission to the study only if the health and mortality history of the test lot conforms to established guidelines (UMESC SOP GEN 132).

The UMESC fish culturist will verify the species of fish in each lot of fish assigned to the study using the identification key in Eddy and Underhill (1978). The current scientific name will be verified with Robins et

al. (1991). All fish will be used without regard to gender. The fish will be raised to an appropriate size (50-600 g) for the study in well water with a nominal temperature of 12°C. The age of fish will range from approximately 9 months to 2 years when they are assigned to the study. The number of fish of each species used in the study will depend on the size of fish available. The primary use of fish will be to acquire control fillet tissue, therefore as fish size increases, the number of fish sacrificed for control tissue will decrease. The conscientious effort will be made to minimize the number of fish used. Less than 30 fish of each species are expected to be used. Three fish of each species will be designated for exposure to isoeugenol for the purpose of generating incurred isoeugenol residues in the fillet tissue. The three fish designated for exposure will be held in a fiberglass tank (291 × 67 × 32 cm, length × width × depth; 624 L, volume) supplied with well water at a flow rate greater than 624 L/h (1tank exchange per hour). The tank will be in a chamber programmed with the current photoperiod. Fish will be held at 12 ± 2°C for at least 1 week before the exposure to biologically incur isoeugenol residues is initiated. The water quality (temperature, pH, and dissolved oxygen) in the holding tank will be monitored once per day while fish are in the holding tank.

4.3. Diet

Fish cultured at the UMESC will be fed Sterling Silver Cup Trout Food (Nelson and Sons, Inc., Murray, Utah), fathead minnows (*Pimephales promelas*), or rainbow trout fry at a rate that maintains fish growth

Table 1. Summary of monthly water analyses for calender year 2001 for well water at the Upper Midwest Environmental Sciences Center, La Crosse, WI.

Characteristic	N	Mean	Range
pH	52	7.92	7.68 - 8.22
Ammonia nitrogen (mg/L)	12	0.09	0.07 - 0.09
Nitrate + nitrite (mg/L)	2	6.3	6.2 - 6.4
Total phosphorous (mg/L)	5	0.082	0.067 - 0.105
Biochemical oxygen demand (mg/L)	12	<4	<2 - 4
Chemical oxygen demand (mg/L)	5	4.9	4.5 - 5.3
Suspended solids (mg/L)	12	<3	<1 - 4
Total organic carbon (mg/L)	5	1	0.96 - 1.2

(Haskell, 1959). The type of feed will be determined by the fish species. Nutrient (e.g. protein, fat, and crude fiber) and contaminant (e.g. organophosphate and organochlorine pesticides, polychlorinated biphenyls, and aflatoxin) analysis of Sterling Silver Cup feed will be conducted by Eurofins Scientific (345 Adams Avenue, Memphis, Tennessee).

4.4. Acquisition of control fillet tissue

At least 10 fish from each species will be euthanized by a blow to the head (UMESC SOP GEN 132, "Care, maintenance, and disposal of aquatic vertebrates") and weighed (step B.4. of UMESC SOP CAP 606, "Methods used to weigh, measure, and mark test animals"). Skin-on fillets will be taken according to the procedures described in step D.9. of UMESC SOP CAP 602, "Dissection and weighing procedures for test fish." Fillets will be hardened in a freezer before homogenizing with dry ice (UMESC SOP CAP 402, "Dry ice homogenization of fish tissue"). The fillet tissue/dry ice matrices will be poured into plastic freezer bags and stored at about -15°C to allow the dry ice to sublime. Homogenized tissue will be stored at <-60°C in plastic freezer bags. Fillet homogenate from one fish will be stored separately from the fillet homogenate of another fish.

4.5. Method to determine isoeugenol concentrations in fillet tissue

Fillet tissue will be weighed (nominal subsample weight, 5 g) in a 25 mL screw cap glass tube and undergo four solvent extractions. Extraction #1: 10 mL of acetonitrile will be added to the sample; the sample will be shaken for 5 min on an automatic wrist action shaker; the sample will be centrifuged at a relative centrifugal field of 950 x *g* for 5 min at ambient temperature; the supernatant will be poured into a

125 mL pear shaped roto-vap flask. Extraction #2: 5 mL of acetonitrile will be added to the sample. The sample will be shaken for 5 min on an automatic wrist action shaker; the sample will be centrifuged at a relative centrifugal field of 950 x *g* for 5 min at ambient temperature; the supernatant will be poured into a 125 mL pear shaped roto-vap flask. Extractions #3 and #4: 5 mL of acetonitrile will be added to the sample. The sample will be shaken for 5 min on an automatic wrist action shaker; the sample will be centrifuged at a relative centrifugal field of 2630 x *g* for 5 min at ambient temperature; the supernatant will be poured into a 125 mL pear shaped roto-vap flask. Acetonitrile will be evaporated from the sample on a rotary evaporator with a vacuum generated with a vacuum pump and a water bath temperature of about 45°C. The sample will be evaporated to about 5 mL before 45 mL of water are added to the sample. The sample will be processed through a Phenomenex Strata phenyl 55 *u*m, 70A, 500 mg, 3 mL solid phase extraction (spe) column. The extract will be pulled through the column at less than 5 mL/min with a vacuum. The column will be eluted with five 1 mL portions of 90:10 methanol:water into a 5 or 10 mL volumetric flask. A portion of the extract will be filtered through a Gelman Acrodisc CR 13 mm syringe filter into an amber glass LC vial. Liquid chromatography parameters similar to the following parameters will be used to determine isoeugenol concentrations in the extracts: isocratic mobile phase, 49% water and 51% acetonitrile; flow rate, 1.5 mL/min; injection volume, 45 *u*L; column temperature, 50°C; guard column, YMC, ODS-A, 5 *u*m, 4.0 x 23 mm; and analytical column, Phenomenex, Synergi Max-RP, 4 *u*m, 4.6 x 250 mm; monitored wavelength, 261 nm. The concentration of isoeugenol in an extract will be determined from the isoeugenol peak area and the linear regression equation developed from a calibration curve created with 5 working solutions of isoeugenol.

4.6. Assuring the integrity of extraction sessions

The following procedures will be implemented when non-fortified tissue samples and tissue samples containing biologically incurred isoeugenol are processed with procedures described in section 4.5. During those analysis sessions, a control fillet tissue sample will be analyzed to evaluate the actuality of isoeugenol contamination in any of the method procedures. In addition, three control fillet tissue samples will be fortified with isoeugenol by applying a small volume (≤100 µL) of an isoeugenol working solution resulting in a nominal tissue concentration of about 50 *u*g/g. Samples will be processed with procedures described in section 4.5. Data generated during the analysis session will be considered acceptable if the percent recovery is between 80 and 110% (accuracy) and the relative standard deviation is <10% (precision).

4.7. Evaluation of chromatographic interferences extracted from control fillet tissue

Three tissue samples from each fish sacrificed for control tissue will be processed with procedures described in section 4.5. Extracts will be evaluated for the presence of compounds that could interfere with the chromatography of isoeugenol leading to errors determining isoeugenol concentrations in fillet tissue. The isoeugenol equivalent concentration of chromatographically interfering peaks will be determined from the peak area of the interfering compound and the linear regression equation developed from a calibration curve created with 5 working solutions of isoeugenol.

4.8. Evaluation of method systemic error

Systemic error (percent recovery) will be assessed by fortifying fillet tissue with a small volume (≤100 µL) of an isoeugenol solution resulting in nominal tissue concentrations of 1, 50, and 100 *u*g/g. Five samples will be prepared for each concentration level. All samples will be processed on the same day with procedures described in section 4.5. Recovery will be calculated by dividing the amount of isoeugenol found by the amount applied to the samples and reported as a percentage.

4.9. Evaluation of method within-day and day-to-day repeatability

Within-day repeatability (precision) will be assessed by fortifying fillet tissue with a small volume (≤100 µL) of an isoeugenol solution resulting in nominal tissue concentrations of 1, 50, and 100 *u*g/g. Five samples will be prepared for each concentration level. All 5 samples from one concentration level will be processed on the same day according to the procedures described in section 4.5. Within-day precision will be reported for each concentration level as the percent relative standard deviation of the isoeugenol concentration in individual samples.

Day-to-day repeatability (precision) will be assessed by fortifying fillet tissue with a small volume (≤100 µL) of an isoeugenol solution resulting in nominal tissue concentrations of 1, 50, and 100 *u*g/g. Five samples will be prepared for each concentration level. All 5 samples from one concentration level will be processed on the same day with procedures described in section 4.5. The process will be repeated four more times on four different days. Day-to-day precision will be reported for each concentration level as

the percent relative standard deviation of the mean isoeugenol concentrations from the 5 days of analyses.

<u>4.10. Evaluation of method repeatability with biologically incurred isoeugenol</u>

AQUI-S® will be prepared by weighing isoeugenol (850 ± 50 mg) and polysorbate 80 (850 ± 50 mg) in a 50 mL screw cap tube. The contents will be shaken on a wrist action shaker for about 15 min. About 5 mL of well water will be added to the flask and the contents shaken for about 15 min. The contents will be shaken throughout the next 45 min with 2 periodic additions of well water (each addition about 5 mL) through that time.

A stainless steel exposure tank (width at one-half the height, 58.4 cm; length at one-half the height, 58.4 cm; height, 35.6 cm) will be filled with about 100 L of 12°C well water. The entire AQUI-S® mixture will be vigorously mixed into the exposure tank with a glass rod for about 1 min. Dissolved oxygen will be measured and three water samples (5 mL) taken from the bath to determine the initial isoeugenol concentration. Within 15 min after preparing the bath, 3 fish in the holding tank will be captured with a net and released into the exposure tank. Fish will remain in the exposure bath for 60 min. At 60 min, fish will be removed from the tank and euthanized by a blow to the head. Dissolved oxygen will be measured in the exposure tank and three water samples (5 mL) taken to determine the final isoeugenol concentration.

Water samples will be applied directly to a conditioned Phenomenex Strata phenyl 55 *u*m, 70A, 500 mg, 3 mL spe column. Water will be pulled through the column at less than 5 mL/min with a vacuum generated by a vacuum pump. The column will be eluted with 5 x 1 mL portions of 90:10 methanol:water into a 5 mL volumetric flask. A portion of the extract will be filtered through a Gelman Acrodisc CR 13 mm syringe filter into a glass LC vial and analyzed for isoeugenol with the LC parameters listed in section 4.5.

Euthanized fish will be rinsed with flowing well water and weighed. The skin-on fillets will be removed from each fish and hardened in a freezer before homogenizing with dry ice. The fillet tissue/dry ice matrices will be poured into plastic freezer bags and stored overnight at about -15°C to allow the dry ice to sublime. The fillet homogenate from one fish will be stored separately from the fillet homogenate of the other fish. The following day, five samples from each fish will be processed with procedures described in section 4.5. Precision will be reported for each fish as the percent relative standard deviation of the isoeugenol concentration in individual samples. The remaining tissue will be stored at <-60°C.

<u>4.11. Evaluation of method detection and quantitation limits</u>

Method sensitivity will be determined by fortifying at least seven subsamples (5 g) of control fillet tissue with isoeugenol by applying a small volume (≤100 µL) of an isoeugenol working solution and processing the samples with procedures described in section 4.5. Estimation of the method detection limit will be calculated as 3*s* and the method quantitation limit will be calculated as 10*s* (Keith et al., 1983) where *s* is the sample standard deviation.

<u>4.12. Evaluation of isoeugenol stability in rainbow trout fillet extract</u>

Control rainbow trout fillet tissue will be fortified with a small volume (≤100 µL) of an isoeugenol solution resulting in nominal tissue concentrations of 1, 50, and 100 *u*g/g. Three tissue samples will be prepared for each concentration level. Samples will be processed with procedures described in section 4.5. The isoeugenol concentration will be determined by LC on the day the solutions are prepared (day 0). Filtered extracts will be stored in amber glass vials on the laboratory bench top exposed to standard room temperature (about 21°C) and fluorescent lighting. Isoeugenol concentrations in the extracts will be determined periodically throughout a 14 day period. The percent concentration change from day 0 will be reported from each analyses.

<u>4.13. Evaluation of isoeugenol stability in rainbow trout fillet tissue</u>

The isoeugenol concentrations determined in fish tissue described in section 4.10 will serve as the day 0 concentrations for this task. Bags of tissue will be allowed to thaw before 3 samples from each fish are processed with procedures described in section 4.5. Tissue will be returned to storage after removing the 3 samples. Tissue will be processed at least 6 times throughout a 6 month period. The percent concentration change from day 0 will be reported from each analyses.

<u>5. Data reporting and statistical analyses</u>

Isoeugenol concentrations in solutions will be reported as *u*g/mL. Isoeugenol concentrations in fillet tissue will be reported as *u*g/g. Accuracy of the method for isoeugenol in fish fillet tissue will be determined by dividing the amount of isoeugenol found in the fortified tissue by the amount of isoeugenol applied. Precision will be reported as the percent relative standard deviation. Where comparisons are to

be made between method accuracy or precision, comparisons will be accomplished by one-sided means comparison test (e.g. Studentized t-test or equivalent; Student, 1908). Comparisons of means will be considered significant if $P \leq 0.05$

Linear regression analysis will be used to determine acceptance of the following null hypotheses: 1) isoeugenol concentration in extracts of incurred residues does not correlate to sample time (i.e., isoeugenol concentration in fillet extract does not decrease) and 2) isoeugenol concentrations in fillet tissue do not correlate to sample time (i.e., isoeugenol concentration in stored fillet tissue does not decrease). Before testing the null hypotheses, isoeugenol concentration data will be tested to determine if the data fit a normal distribution (Shapiro and Wilk, 1965). The assumption of equal variance will be assessed graphically by reviewing plots of predicted and residual isoeugenol concentrations versus observed isoeugenol concentrations. The model for the first null hypotheses will compare isoeugenol concentration in fillet extract (hypothesis 1) with sample time to identify isoeugenol degradation rates. The model for the second null hypothesis will compare storage time (e.g., time post-sample collection) with fillet tissue isoeugenol concentrations to determine if isoeugenol in fillet tissue degrades while tissue is stored at $<-70^{\circ}C$. Model parameters will be estimated with least squares regression (Proc GLM; SAS, 2000) for repeated measures and the null hypotheses accepted if the slope of the model's regression line is not significantly different from zero ($P \geq 0.05$). Model fit will be assessed on the overall model significance and model r^2. Fillet concentrations of isoeugenol will be natural log transformed before model parameters are estimated.

6. Safety

Safety precautions described in the Material Safety Data Sheets of the chemicals used for this study will be followed. Standard laboratory safety equipment such as gloves, lab coats, and eye protection will be made available to analysts and worn when necessary.

7. Study records

Study records will be maintained with the following procedures described in UMESC SOP GEN 002, “Procedures to meet good laboratory practice requirements”: “*All data generated in the study will be recorded in bound laboratory notebooks or kept in file folders (SOP No. GEN 008, “Laboratory data books and recording of data”). All data sheets, file folders, laboratory notebooks and computer disks will be encoded with the study number when the data are generated and stored in secure files (SOP No. GEN 008). Raw data, laboratory notebooks and electronic files will be filed in the archives (SOP No. GEN 007, “Archives management for regulated studies”) of the Upper Midwest Environmental Sciences Center, La Crosse, Wisconsin, before the final report is signed by the Study Director. The final report will then be signed and archived.”*

8. Good laboratory practices

Good laboratory practices will be implemented following procedures described in UMESC SOP GEN 002, “Procedures to meet good laboratory practice requirements”: “*Data collection, storage, and retrieval procedures for the study will be conducted in compliance with FDA regulations for Good Laboratory Practices (21 CFR, Part 58). The study protocol and progress of the study will be reviewed at the start of the study and periodically throughout the study by the Quality Assurance Unit (QAU). The Study Director has the responsibility of ensuring that all procedures used in conjunction with the study conform with Good Laboratory Practices.*

All deviations from this approved protocol will be documented in the laboratory notebook and reviewed by the Study Director, who will make a judgement on the impact of the deviations. The Study Director will notify the QAU and Management as soon as possible, in writing, of any deviations to the protocol, including their impact on the study.

Proposed amendments to the protocol shall be brought to the attention of the sponsor and Management. When the Study Director, sponsor and Management agree verbally, the study can proceed with the change. As soon as possible, the Study Director will then prepare a written protocol amendment that is signed by the Study Director, Sponsor, Branch Chief, Center Director, and QAU. The amendment then becomes an official part of the protocol.”

9. References

Eddy, S. and J.C. Underhill. 1978. Northern Fishes, third edition, University of Minnesota Press, Minneapolis, MN. 215 pages.

Haskell, D.C. 1959. Trout growth in hatcheries. *New York Fish and Game Journal* **6(2)**, 204-237.

Keith, W. Crummett, J. Deegan, R.A. Libby, J.K. Taylor, and G. Wentler. 1983. Principles of environmental analysis. Analytical Chemistry, 55:2210-2218.
Robins, C.R., R.M. Bailey, C.E. Bond, J.R. Brooker, E.A. Lachner, R.N. Lea, and W.B. Scott. 1991. Common and Scientific names of fishes from the United States and Canada, fifth edition, American Fisheries Society, Bethesda MD. 183 pages.
SAS Institute Inc., 2000. Version 8.01 Edition. Cary, NC: SAS Institute Inc.
Shapiro, S.S., Wilk, M.B. 1965. An analysis of variance test for normality (complete samples). Biometrika. 52, 591-611.
Student. 1908. The probable error of a mean. Biometrika, 6:1-25

Submit a final report (Objective 3)

A final report describing the results of the method validation will be submitted to CVM. The report format will be consistent with the format of reports previously submitted by the project leader to CVM describing method validation results. All data from the study will be submitted to the UMESC archives. A progress report describing the accumulation of activities through August 31, 2006 will be submitted to the Director of the North Central Regional Aquaculture Center by October 31, 2006.

Gain from CVM approval of the proposed method (Objective 4)

CVM will review the final report and provide comments to the project leader. The project leader will address all concerns and conduct additional work if necessary to gain CVM acceptance of the data. The project leader will revise or append data to the original report and resubmit the report to CVM.

FACILITIES

The UMESC is equipped with state of the art computers and word processing, statistical, and spreadsheet software programs. During the construction of the protocol, the project leader can incorporate comments from the team of professionals built by the UMESC to coordinate and conduct aquaculture drug research, including chemists, physiologists, fish biologists, fish culturists, and general biologists.

Each person is considered an expert in their particular specialty field. Because aquaculture drugs will be used on a human food source, each person was trained and certified to implement FDA's Good Laboratory Practice guidelines. The scientists at the UMESC are equipped with state of the art equipment including an expansive aquaculture facility specifically geared for rearing a wide range of fish species, environmental chambers that allow researchers to conduct a variety of acute and chronic studies with fish in a controlled environment, water conditioning capabilities that allow the chemical composition and water temperature to be tailored to meet precise research needs, spacious indoor wet laboratory areas allow researchers to fabricate virtually any test system to suit their diverse experimental designs. Multiple analytical laboratories with chemical fume hoods allow for a variety of chemical analyses. Equipment is available for analyzing radioisotopes, analytical equipment that can detect chemicals at the parts per trillion level, and a wide variety of general laboratory equipment including analytical balances, ultra cold freezers, digital image microscopes, tissue homogenizers, rotary evaporators, clinical and ultra centrifuges, and liquid chromatography systems.

REFERENCES

See "Reference" section in the text associated with the **PROCEDURES, Objective 2** section.

PROJECT LEADER

State	Name	Institution
Wisconsin	Jeffery R. Meinertz	USGS Upper Midwest Environmental Sciences Center

PARTICIPATING INSTITUTION AND PRINCIPAL INVESTIGATOR

USGS Upper Midwest Environmental Sciences Center
Jeffery R. Meinertz

UNITED STATES DEPARTMENT OF AGRICULTURE OMB Approved 0524-0039
COOPERATIVE STATE RESEARCH, EDUCATION, AND EXTENSION SERVICE Expires 03/31/2004

BUDGET

ORGANIZATION AND ADDRESS USGS, Upper Midwest Environmental Sciences Center 2630 Fanta Reed Road La Crosse, WI, 54603	USDA AWARD NO. Year 1: Objectives 1-4			
PROJECT DIRECTOR(S) Jeffery R. Meinertz	Duration Proposed Months: _12_ **Funds Requested by Proposer**	Duration Proposed Months: ____ **Funds Approved by CSREES** (If different)	Non-Federal Proposed Cost-Sharing/ Matching Funds (If required)	Non-federal Cost-Sharing/ Matching Funds Approved by CSREES (If Different)

	CSREES FUNDED WORK MONTHS: Calendar	Academic	Summer	Funds Requested by Proposer	Funds Approved by CSREES	Non-Federal Proposed Cost-Sharing	Non-federal Cost-Sharing Approved
A. Salaries and Wages 1. No. of Senior Personnel				$60,580			
a. _1_ (Co)-PD(s)	10						
b. _1_ Senior Associates	6			$32,292			
2. No. of Other Personnel (Non-Faculty) a. ___ Research Associates-Postdoctorates							
b. ___ Other Professionals							
c. ___ Paraprofessionals							
d. ___ Graduate Students							
e. ___ Prebaccalaureate Students							
f. ___ Secretarial-Clerical							
g. ___ Technical, Shop and Other							
Total Salaries and Wages →				$92,872			
B. Fringe Benefits (If charged as Direct Costs)				$28,064			
C. Total Salaries, Wages, and Fringe Benefits (A plus B) →				$120,936			
D. Nonexpendable Equipment (Attach supporting data. List items and dollar amounts for each item.)							
E. Materials and Supplies				$5,000			
F. Travel							
G. Publication Costs/Page Charges							
H. Computer (ADPE) Costs							
I. Student Assistance/Support (Scholarships/fellowships, stipends/tuition, cost of education, etc. Attach list of items and dollar amounts for each item.)							
J. All Other Direct Costs (In budget narrative, list items and dollar amounts and provide supporting data for each item.)				$4,000			
K. Total Direct Costs (C through I) →				$129,936			
L. F&A/Indirect Costs. (If applicable, specify rate(s) and base(s) for on/off campus activity. Where both are involved, identify itemized costs in on/off campus bases.)							
M. Total Direct and F&A/Indirect Costs (J plus K) →				$129,936			
N. Other →							
O. Total Amount of This Request →				$129,936			

P. Carryover -- (If Applicable) . . . . **Federal Funds: $** **Non-Federal funds: $** **Total $**

Q. Cost Sharing/Matching (Breakdown of total amounts shown in line O)		
Cash (both Applicant and Third Party) →		
Non-Cash Contributions (both Applicant and Third Party) →		

NAME AND TITLE (Type or print)	**SIGNATURE** (required for revised budget only)	**DATE**
Project Director		
Authorized Organizational Representative		
Signature (for optional use)		

According to the Paperwork Reduction Act of 1995, an agency may not conduct or sponsor, and a person is not required to respond to a collection of information unless it displays a valid OMB control number. The valid OMB control number for this information collection is 0524-0039. The time required to complete this information collection is estimated to average 1.00 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing the reviewing the collection of information.

Form CSREES-2004 (12/2000)

BUDGET EXPLANATION FOR THE UPPER MIDWEST ENVIRONMENTAL SCIENCES CENTER

A. Salaries and Wages. Ten months for Principal Investigator and six months for senior associate.

B. Fringe Benefits. The UMESC senior and project director fringe benefit is 30%.

E. Materials and Supplies. These funds will be used to acquire expendable supplies used during the extraction process and will include solvents, guard columns, solid phase extraction columns, syringes, syringe filters, and disposable pipettes.

J. Other Direct Costs. These funds will be used to partially support the maintenance contract for the high performance liquid chromatograph.

SCHEDULE FOR COMPLETION OF OBJECTIVES

Objective 1: Initiated during January 2006 and completed during April 2006.

Objective 2. Initiated during April 2006 and completed during October 2006.

Objective 3. Initiated during September 2006 and completed during October 2006.

Objective 4. Initiated during November 2006 and completed during December 2006.

LIST OF PRINCIPAL INVESTIGATOR

Jeffery R. Meinertz, USGS, Upper Midwest Environmental Sciences Center

VITA

Jeffery R. Meinertz
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EDUCATION

B.S. 1985 University of Wisconsin-La Crosse, Biology
M.S. 1989 University of Wisconsin-La Crosse, Biology

POSITIONS

Research Physiologist (1990-present), Upper Midwest Environmental Sciences Center, La Crosse, Wisconsin
Physical Science Technician (Chemistry and Physiology) (1988-1990), National Fisheries Research Center, La Crosse, Wisconsin
Quality Control Assistant Manager (1988), Seafreeze Ltd., Seattle, Washington

SCIENTIFIC AND PROFESSIONAL ORGANIZATIONS

American Fisheries Society

SELECTED PUBLICATIONS

Idowu, O.R., P.J. Kijak, J.R. Meinertz, and L.J. Schmidt. 2004. Development and validation of a gas chromatography/mass spectrometry procedure for the confirmation of para-toluenesulfonamide in edible fish fillet tissue. Journal of AOAC International 87:1098-1108.

Dawson, V.K., J.R. Meinertz, L.J. Schmidt, and W.H. Gingerich. 2003. A simple analytical procedure to replace HPLC for monitoring of treatment concentrations of chloramine-T on fish culture facilities. Aquaculture 217:61-72.

Meinertz, J.R., G.R. Stehly, W.H. Gingerich, and S.L. Greseth. 2001. Performance of a proposed determinative method for p-TSA in rainbow trout fillet tissue and bridging the proposed method with a method for total chloramine-T residues in rainbow trout fillet tissue. Journal of AOAC International 84:1332-1336.

Meinertz, J.R., G.R. Stehly, and W.H. Gingerich. 1999. Liquid chromatographic determination of benzocaine and N-acetylbenzocaine in the edible fillet tissue from rainbow trout. Journal of Chromatography A 855:255-260.

Meinertz, J.R., L.J. Schmidt, G.R. Stehly, and W.H. Gingerich. 1999. Liquid chromatographic determination of para-toluenesulfonamide in the edible fillet tissue from three species of fish. Journal of AOAC International 82:1064-1070.

Meinertz, J.R., G.R. Stehly and W.H. Gingerich. 1998. Liquid chromatographic determination of oxytetracycline in edible fish fillets from six species of fish. Journal of AOAC International 81:702-708.