FINAL REPORT

Study Title

The Uterotrophic Assay with
Avobenzone, Ensulizole, Homosalate, and Padimate-O

ILS Project-Study Numbers

N135-247

Guideline Reference Number

OPPTS 890.1600

Author

[redacted]

Performing Laboratory

Integrated Laboratory Systems, Inc.
601 Keystone Park Drive, Suite 100
Durham, NC 27713 USA

Sponsor

National Institute of Environmental Health Sciences
P.O. Box 12233
Research Triangle Park, NC 27709 USA

Date of Completion

07 November 2012

STATEMENT OF NO DATA CLAIM OF CONFIDENTIALITY

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Sponsor: National Institute of Environmental Health Sciences

Sponsor Representative: [redacted]

Title: Contract Officer Technical Representative

Signature: [redacted]

Date: 11/7/12

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

This study was conducted in accordance with U.S. EPA Good Laboratory Practice Standards, 40 CFR §160 with the following exceptions:

17-α Ethinyl estradiol was not analyzed as stated in 40 CFR 160.113(a)(1) of the U.S. EPA GLP requirements, a positive response in the test system following 17α-ethinyl estradiol administration was evident following statistical analysis of the tissue weights.

Dose formulation analyses were performed at the following laboratories at the request of the Sponsor: analysis for Avobenzone with [redacted] as the Study Director at MRIGlobal (Kansas City, MO), analysis for Ensulizole, Homosalate, and Padimate-O with [redacted] as the Study Director all at Research Triangle Institute, International (Research Triangle Park, NC).

Study Director: [redacted]

Signature: [redacted] Date: 11-7-12

Typed Name of Laboratory: Integrated Laboratory Systems, Inc.

Typed Name of Study Monitor/Sponsor/Subm[redacted]

Signature: [redacted] Date: 11/7/12

Typed Name of Company: National Institute of Environmental Health Sciences

[redacted] Date: 11-7-12

Study Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

QUALITY ASSURANCE INSPECTION STATEMENT

Laboratory Project ID - Study No.: N135-247

Study Title: The Uterotrophic Assay with Avobenzone, Ensulizole, Homosalate, and Padimate-O

This study was inspected by one or more persons of the Quality Assurance Unit of ILS, Inc., Research Triangle Park, NC, US, and written status reports were submitted on the following dates:

Inspection/Audit:	Date(s) Performed:	Dates Reported to Study Director / Management:
Study Protocol	20 March 2012	20 March 2012/20 March 2012
Dose Administration	03 April 2012	03 April 2012/03 April 2012
Necropsy	05 April 2012	05 April 2012/05 April 2012
Data Audit	17-18 April 2012	18 April 2012/19 April 2012
Draft Report	31 May - 1 June 2012; 08 August 2012	03 June 2012/11 June 2012 08 August 2012/08 August 2012
Final Report	06 November 2012	06 November 2012/ 06 November 2012

[redacted]

Quality Assurance Auditor

07-Nov-2012

Date

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Study Summary

Ovariectomized adult female rats were orally administered corn oil (vehicle control), 320 or 1000 mg/kg/day Avobenzone, Ensulizole, Homosalate, or Padimate-O or 0.5 mg/kg/day 17α-ethinyl estradiol (positive control) for 3 consecutive days and then humanely euthanized. Body weights and clinical observations were performed daily. At termination, uteri were excised and wet and blotted weights recorded.

Following administration of 17α-ethinyl estradiol, wet and blotted uterine weights were significantly increased compared to vehicle controls indicating a positive response in the animal model.

Administration of Padimate-O and Avobenzone significantly decreased body weight gain at 1000 mg/kg/day, but not at 320 mg/kg/day compared to vehicle control animals. Administration of Homosalate significantly decreased final body weight at 1000 mg/kg/day, but not 320 mg/kg/day. Body weight gain was not significantly different at either dose level of Homosalate. Administration of Ensulizole did not affect body weights or body weight gain compared to vehicle controls. Uterine weights (wet and blotted) were not significantly different at either dose level of Avobenzone, Ensulizole, Homosalate, or Padimate-O compared to the vehicle control.

Oral administration of Avobenzone, Ensulizole, Homosalate, or Padimate-O, up to the limit dose level of 1000 mg/kg/day, were not estrogenic in the ovariectomized rat model Uterotrophic Assay (OPPTS 890.1600).

INTRODUCTION

1.1 Study Title

The Uterotrophic Assay with Avobenzone, Ensulizole, Homosalate, and Padimate-O

1.2 Laboratory Project Identification

ILS Project No.-Study No.: N135-247

1.3 Background

The Endocrine Disruptor Screening Program (EDSP) reflects a two-tiered approach to implement the statutory testing requirements of FFDCA section 408(p) (21 U.S.C. 346a). The U.S. EPA will use the data collected under the EDSP, along with other information to determine if a pesticide chemical, or other substances, may pose a risk to human health or the environment due to disruption of the endocrine system.

EDSP Tier 1 screening assays will be used to identify substances that have the potential to interact with the estrogen, androgen, or thyroid hormone systems (test guidelines in the OPPTS 890 series). The determination of the potential of each test substance's endocrine activity will be made on a weight-of-evidence basis taking into account data from the Tier 1 assays and other scientifically-relevant information available. The fact that a substance may interact with a hormone system, however, does not mean that when the substance is used it will cause adverse effects in humans or ecological systems. The Uterotrophic Assay (OPPTS 890.1600) is used to screen substances for estrogenicity and is 1 of 4 *in vivo* mammalian assays in the EDSP Tier 1 battery of assays.

1.4 Purpose of the Study

The purpose of this Uterotrophic Assay was to screen four test substances selected by the National Toxicology Program for their estrogenicity using the ovariectomized rat model (OPPTS 890.1600).

1.5 Sponsor

National Institute of Environmental Health Sciences (NIEHS)
P.O. Box 12233
Research Triangle Park, NC 27709

NIEHS Investigator

[redacted]
Telephone No.: [redacted]

[redacted] [redacted]

Study Monitor

[redacted]
Contract Officer Technical Representative
Telephone No.: [redacted]
E-mail: [redacted]

1.6 Testing Facility **Integrated Laboratory Systems, Inc. (ILS)**

Shipping Address: 601 Keystone Park Drive, Suite 100
Durham, NC 27713

Mailing Address: P.O. Box 13501
Research Triangle Park, NC 27709

Study Director

[redacted]
Telephone No.: [redacted]
Facsimile No.: [redacted]
E-mail: [redacted]

1.7 Study Dates

Study Initiation Date: 26 March 2012
Animal Arrival Date: 26 March 2012
Experimental Start Date: 02 April 2012
Experimental End Date: 06 April 2012

TEST SUBSTANCE

2.1 Test Substance: 2-Phenyl-5-benzimidazolesulfonic Acid (Ensulizole)

CAS No. 27503-81-7

Source: Sigma-Aldrich

Lot/Batch No.: 05117JE

ILS Repository No.: 12-25

Formula: $C_{13}H_{10}N_2O_3S$

Description: White Powder

Purity: 99.6%

Dose Formulation:	Test substance was prepared one time during the study at ILS. Ensulizole formulations in corn oil were prepared at concentrations of 64 and 200 mg/ml and dispensed into 15 mL amber vials that were used for daily dosing throughout the study.
Storage:	
Test Substance:	Ambient temperature protected from light
Dose Formulation:	Ambient temperature protected from light
Stability:	
Dose Formulation:	Ensulizole in corn oil stored at ambient temperature was shown to be stable for 43 days (Blake, 2012a).

2.2 Test Substance **Butyl-methoxydibenzoylmethane (Avobenzone)**

CAS No.	70356-09-1
Source:	Universal Preserv-A-Chem, Inc.
Lot/Batch No.:	L802809
Expiration:	14 June 2012
ILS Repository No.:	12-19
Formula:	$C_{20}H_{22}O_3$
Description:	Off white to yellowish, crystalline powder
Purity:	98.3%
Dose Formulation:	Test substance was prepared one time during the study at ILS. Avobenzone formulations in corn oil were prepared at concentrations of 64 and 200 mg/ml and dispensed into 15 mL amber vials that were for daily dosing throughout the study. Dose concentrations were adjusted to correct for purity of Avobenzone.

Storage:

Test Substance: Ambient temperature protected from light

Dose Formulation: Ambient temperature protected from light

Stability:

Dose Formulation: Avobenzone in corn oil stored at ambient temperature was shown to be stable for 42 days (Aillon, 2012a).

2.3 Test Substance **3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)**

CAS No. 118-56-9

Source: Spectrum Laboratory Products Inc

Lot/Batch No.: YT0976

ILS Repository No.: 12-24

Formula: $C_{16}H_{22}O_3$

Description: Colorless to light yellow liquid

Purity: 99.3%

Dose Formulation: Corn Oil

Dose Formulation: Test substance was prepared one time at ILS during the study. Homosalate formulations in corn oil were prepared at concentrations of 64 and 200 mg/ml and dispensed into 15 mL amber vials that were used for daily dosing throughout the study. Dose concentrations were adjusted to correct for purity of homosalate.

Storage:

Test Substance: Ambient temperature

Dose Formulation: Ambient temperature protected from light

Stability:

Dose Formulation: Homosalate in corn oil stored at ambient temperature was shown to be stable for 42 days (Blake, 2012c).

2.4 Test Substance **2-Ethylhexyl-P-Dimethyl-Aminobenzoate (Padimate-O)**

CAS No. 21245-02-3

Source: Sigma-Aldrich Company

Lot/Batch No.: MKBF0590V

ILS Repository No.: 12-26

Formula: $C_{17}H_{27}NO_2$

Description: Colorless liquid

Purity: 98.1%

Dose Formulation: Test substance was prepared one time at ILS during the study. Padimate-O formulations in corn oil were prepared at concentrations of 64 and 200 mg/ml and dispensed into 15 mL amber vials that were used for daily dosing throughout the study. Dose concentrations were adjusted to correct for purity of Padimate-O.

Storage:

Test Substance: Ambient temperature

Dose Formulation: Ambient temperature protected from light

Stability:

Dose Formulation: Padimate-O in corn oil stored at ambient temperature was shown to be stable for 43 days (Blake, 2011).

2.5 Reference Substance: **17α-Ethinyl Estradiol**

CAS No. 57-63-6

Source: Sigma Aldrich

Lot/Batch No.:	090M1241V
ILS Repository No.:	11-40
Formula:	$C_{20}H_{24}O_2$
Description:	White powder
Purity:	≥98%
Expiration Date:	February 2012
Dose Formulation:	ILS prepared 17α-ethinyl estradiol in corn oil once at a dose level of 0.01 mg/mL and dispensed into amber vials to be used daily during the study.
Storage:	
Reference Substance:	Room temperature and protected from light
Dose Formulation:	Stored between 1-10°C
Stability:	
Dose Formulation:	17α-Ethinyl estradiol in corn oil, stored between 1-10°C, is stable for 42 days (Messer, 2002).

2.6 Vehicle **Corn Oil**

CAS No.:	8001-30-7
Source:	MP Biomedicals, LLC
Lot/Batch No.:	7862K
ILS Repository No.:	11-121
Formula:	$C_{27}H_{50}O_6$
Description:	Yellow oil
Storage:	
Vehicle:	Room Temperature

Justification: Corn oil was selected based on the solubility of the test substances.

2.7 Archival Samples

Approximately a 1 g sample of the neat test substance, approximately 1 mg of the reference substance, and 1 mL of the corn oil (vehicle) and dose formulations for each preparation will be stored between 0 and -30°C until acceptance of the final report; after acceptance of the report by the Sponsor archival samples of dose formulations only will be discarded. The archival samples of test and reference substances will be maintained by ILS for 5 years following finalization of the study report.

2.8 Dose Formulation Analysis

Dose formulations were prepared at ILS and then sent and analyzed at Midwest Research Institute (Kansas City, MO) and Research Triangle Institute, International (Research Triangle Park, NC) in accordance with GLP regulations as promulgated by the U.S. EPA (40 CFR Part 160).

Avobenzone:
Midwest Research Institute
[redacted]
Program: NTP Chemistry Support
425 Volker Boulevard
Kansas City, MO 64110-2299

Ensulizole, Homosalate, and Padimate-O:
Research Triangle Institute International
[redacted]
Materials Handling Facility
East Institute Drive
Research Triangle Park, NC 27709

Samples of dose formulations prepared on 22 March 2012 were collected from the top, middle, and bottom of the formulation and sent to Midwest Research Institute or Research Triangle Institute for analysis. Midwest Research Institute or Research Triangle Institute analyzed samples in duplicate for concentration and homogeneity (Appendix II).

Concentration results were acceptable if the mean concentration was within 10% of the target concentration for Avobenzone, Homosalate, and Padimate-O formulations. Concentration results were acceptable if the mean concentration was within ≤15% of the target concentration for Ensulizole formulations. Homogeneity results were acceptable if the coefficient of variation was less than ≤5% of the target concentration for Avobenzone, Homosalate, and Padimate-O formulations. Homogeneity results were acceptable if the coefficient of variation was less than ≤15% of the target concentration for Ensulizole formulations.

EXPERIMENTAL DESIGN

3.1 Test System

Species:	Rat, *Rattus norvegicus*
Strain:	Sprague-Dawley Crl:CD®(SD) IGS
Source:	Charles River Laboratories International, Inc. (Raleigh, NC)
Number/Sex:	80/Ovariectomized females. Surgical manipulation was performed by Charles River Laboratories International, Inc.
Date of birth:	06 February 2012
Age at arrival:	Postnatal Day (PND) 49 Note: PND is the date of birth
Acclimation:	Animals were acclimated in the study room for 7 days
Age at dose administration:	PND 56/57
Weight at dose administration:	187.9 – 264.3 grams
Identification:	Each animal was uniquely identified by ear punch prior to dose administration. Until the animals were ear punched, they were identified by the temporary numbers located on the animal's cage.
Justification:	Animal model used is in accordance with OPPTS 890.1600: Uterotrophic Assay (U.S. EPA, 2009).

3.2 Animal Husbandry

All procedures were in compliance with the Animal Welfare Act Regulations, 9 CFR 1-4 and animals were handled and treated according to the *Guide for the Care and Use of Laboratory Animals* (ILAR, 2011).

Housing (pre-allocation):	1 per cage
Housing (post allocation):	2 per cage
Cage Changes:	At least twice per week
Cage Type:	Polycarbonate with micro-isolator top
Cage Size:	23 cm wide by 44 cm long (1012 cm^2 area) and 21 cm high
Bedding:	Absorbent heat-treated hardwood bedding (Northeastern Products Corp., Warrensburg, NY)
Diet:	Teklad Global 16% Protein Rodent Diet (Teklad Diets, Madison, WI) *ad libitum* Prior to shipment rats were given Autoclaved Purina5L79 Rat and Mouse diet *ad libitum* at Charles River Laboratories International, Inc. A copy of the diet composition is included in the raw data.
Analysis:	The manufacturer's analytical results are included in the raw data and reviewed prior to animal arrival. The total genistein equivalent of genistein plus daidzein (as described by Owens et al., 2003) was determined to be 3.8 µg/g.
Water:	Reverse osmosis treated tap water (City of Durham, NC) *ad libitum*
Supplied:	Glass water bottles with stainless steel sipper tubes
Analysis:	The results of the current annual comprehensive chemical analyses of water from National Testing Laboratories, Inc. (Cleveland, OH) were reviewed prior to initiation of the study and are included in the raw data.

Water Bottle Changes: At least once per week

Animal Room Conditions:

Temperature: 22.4-25.2°C (See protocol deviation 1)

Humidity 29-58% (See protocol deviation 1)

Lighting: 12/12 hour light/dark cycle

Cleaning: Sanitized within 5 days of receipt

Enrichment: None

STUDY DESIGN

4.1 Allocation

The animals were assigned to a dose group using a procedure that stratifies animals across groups by body weight such that mean body weight of each group was not statistically different from any other group using analysis of variance [ANOVA, Statistical Analysis System (SAS) version 9.2, SAS Institute, Cary, NC]. Only clinically-healthy animals were used in the study.

4.2 Group Designation

Table 1. Group Number, Animal Identification, Dose Group and Level

Group Number	Animal Identification	Dose Group	Test Substance Dose Level (mg/kg/day)
1	01-08	Vehicle Control (corn oil)	0
2	09-16	Homosalate	320
3	17-24	Homosalate	1000
4	25-32	Padimate-O	320
5	33-40	Padimate-O	1000
6	41-48	Avobenzone	320
7	49-56	Avobenzone	1000
8	57-64	Ensulizole	320
9	65-72	Ensulizole	1000
10	73-80	17α-Ethinyl estradiol	0.05

4.3 Dose Administration

The dose formulations were administered via oral gavage at a dose volume of 5 mL/kg body weight. The dose formulations were administered on a staggered start for 3 consecutive days. The first 4 animals from each group were dosed beginning on day 1 of study, and the second 4 animals from each group were dosed on day 2 of study. Dosing occurred 24-hours (± 2 hours) from the previous dose. The dosing sequence was stratified across dose groups; 1 animal from each group and then repeated until all animals are dosed. Date, time, volume, and administered amount (mg/kg/day) of test substances and 17α-ethinyl estradiol are listed in Appendix III.

4.3.1. Justification of Route of Administration

Selection of the route of administration is in accordance with OPPTS 890.1600: Uterotrophic Assay (U.S. EPA, 2009).

4.3.2. Justification of Dose Levels

Selection of the dose levels for each test substance was based on the available EC_{50} and OPPTS 890.1600 guidelines which state "to select doses that ensure animal survival and that are without significant toxicity or distress to the animals after 3 consecutive days of chemical administration up to a maximum dose of 1000 mg/kg/d".

A dose level of 0.05 mg/kg/day 17α-ethinyl estradiol was used based on historical data from studies conducted at ILS, Inc.

4.3.3. Disposal of Dose Formulations

Dose formulations were disposed of as hazardous material following dose administration each day.

4.4 In-Life Animal Observations

Mortality/Moribundity:	Twice daily on weekdays, once daily on weekends/holidays.
Clinical Observations:	Observed within 2 days of arrival, again for allocation of animals to study groups, daily prior to dose administration, and prior to euthanasia.
Cage-side Observations:	Observed 3-hours (± 30 minutes) following dose administration.
Body Weights:	Collected within 2 days of arrival, again for allocation of animals to study groups, daily prior to dose administration, and prior to euthanasia.

4.5 Termination

Scheduled:	Twenty-four hours (± 2 hours) after the final dose administration, animals were humanely euthanized by carbon dioxide (CO_2) asphyxiation with death confirmed by cervical dislocation, in the same order as they were dosed.
Tissue Collection:	The urinary bladder and ureters were removed from the ventral and lateral sides of the uterus and vagina. The uterus and vagina were removed from the body, and excess fat and connective tissue were trimmed away. The vagina was removed from the

uterus below the cervix, so that the cervix remained with the uterine body.

The ends of the uterine horns were examined for the presence of any ovarian tissue. None was present at the time of necropsy.

Gross observations of the uterus were recorded.

Tissue Weights: The uterus was weighed to the nearest 0.0001 g. The uterus was then pierced and blotted to remove the luminal contents and weighed (blotted) to the nearest 0.0001 g.

1. Wet uterus
2. Blotted uterus

4.6 Statistical Analysis

Descriptive statistics (mean and standard deviation) were calculated using MS Excel. Final body weight, body weight gain, and tissue weights were analyzed using SAS version 9.2 (SAS Institute, Cary, NC). Studentized residual plots were used to detect possible outliers and Levene's test was used to assess homogeneity of variance.

Final body weight, body weight gain, and uterine weights were analyzed by one-way ANOVA followed by pair-wise comparisons using a Dunnett's one tailed t test (uterine weights) and Dunnett's two tailed t test (final body weight and body weight gain). Statistically-significant effects were reported when $p < 0.05$.

Positive control animals (17α-ethinyl estradiol) were compared to vehicle controls using the t test procedure. Statistically-significant effects were reported when $p<0.05$.

4.7 Record Retention

All original data [including the original signed study protocol and all amendments (if any), test substance information, animal receipt records, animal caretaker records, observations, body weight records, clinical observations, etc.] and the original final report will be transferred to the National Toxicology Program Archives following finalization of the study report to the address below:
NTP Archives
[redacted]
615 Davis Drive, Suite 300
Durham, NC 27713

RESULTS

5.1 Dose Formulation Analysis

The concentration and homogeneity of all test substance dose formulations were within the acceptable criteria (Appendix II).

Table 2. Dose Formulation Results

Test Substance	Nominal Dose Concentration (mg/mL)	Actual Dose Concentration* (mg/mL) [Percent from Nominal]	Percent CV (Homogeneity)	Nominal Dose Level (mg/kg/day)	Actual Dose Level (mg/kg/day)
Homosalate	64	61.4 [4.1]	1.8	320	307.0
Homosalate	200	194.0 [3.2]	3.5	1000	970.0
Padimate-O	64	62.9 [1.7]	3.1	320	314.5
Padimate-O	200	191.0 [4.5]	3.1	1000	955.0
Avobenzone	64	63.3 [1.1]	1.0	320	316.5
Avobenzone	200	209.5 [4.8]	0.8	1000	1047.5
Ensulizole	64	56.6 [11.6]	11.7	320	283.0
Ensulizole	200	189.0 [5.5]	10.3	1000	945.0

Sources: Allion (2012b); Blake (2012b,d,e)
Abbreviations: CV – coefficient of variation

5.2 In-Life Animal Observations

Mortality/Moribundity

All animals survived to the scheduled study termination with no animals showing signs of moribundity.

Clinical Observations

Animals administered vehicle control (corn oil), Homosalate, Padimate-O, Avobenzone, or Ensulizole exhibited no abnormal clinical signs (Appendix IV).

Cage-side Observations

Animals administered vehicle control (corn oil), Homosalate, Padimate-O, Avobenzone, or Ensulizole exhibited no abnormal clinical signs (Appendix IV).

Body Weights

Group mean initial and final body weights and weight changes for animals euthanized following 3 consecutive days of administration are presented in Table 3. Individual animal data are listed in Appendix V.

The final body weight of rats administered 1000 mg/kg/day Homosalate was significantly decreased compared to vehicle controls. The body weight gain of female rats administered 1000 mg/kg/day Padimate-O or Avobenzone was significantly decreased compared to the vehicle control group. The body weight gain of female rats administered 50 mg/kg/day 17-α estradiol was significantly decreased compared to the vehicle control group.

The final body weight (and body weight gain) of rats administered Padimate–O, Avobenzone (320 mg/kg/day) and Ensulizole (320 and 1000 mg/kg/day) was not statistically different compared to the vehicle control group. Body weight gain of rats administered 1000 mg/kg/day Homosalate was not different than vehicle controls.

Table 3. Group Mean Initial, Final, and Body Weight Changes

Dose Group	Dose Level (mg/kg/day)	n	Initial Body Weight Mean (g) ± SD	Final Body Weight Mean (g) ± SD	Body Weight Change Mean (g) ± SD
Vehicle Control (Corn Oil)	0	8	229.2 ± 20.6	244.6 ± 21.9	15.4 ± 2.7
Homosalate	320	8	231.9 ± 20.6	241.5 ± 17.8	9.6 ± 4.7[¥]
Homosalate	1000	8	225.7 ± 22.7	**218.7 ± 18.8***	-7.0 ± 12.6[¥]
Padimate-O	320	8	228.3 ± 19.7	242.0 ± 18.8	13.7 ± 3.6
Padimate-O	1000	8	231.7 ± 19.2	233.4 ± 23.8	**1.7 ± 9.1***
Avobenzone	320	8	231.6 ± 16.6	241.6 ± 17.1	10.0 ± 5.3
Avobenzone	1000	8	231.5 ± 15.8	239.3 ± 17.4	**7.8 ± 5.2***
Ensulizole	320	8	228.9 ± 16.1	245.8 ± 17.0	16.9 ± 3.1
Ensulizole	1000	8	231.3 ± 19.6	246.2 ± 21.5	15.0 ± 4.8
17α-Ethinyl estradiol	0.05	8	231.4 ± 18.2	231.5 ± 17.2	**0.2 ± 6.1**[†]

Abbreviation: SD - standard deviation

*Statistically significant ($p<0.05$) compared to the vehicle control

†17α-Ethinyl estradiol statistically significant ($p<0.05$) compared to the vehicle control

[¥]Inverse Transformed

5.3 Necropsy

Uterine Weights

Group mean wet and blotted uterine weights for animals euthanized following 3 consecutive days of test substance administration are presented in Table 4. Individual animal tissue weight data are listed in Appendix VI.

Administration of Homosalate, Padimate-O, Avobenzone, or Ensulizole did not affect either wet or blotted uterine weights compared to the vehicle control (corn oil) group. The positive control, 17α-ethinyl estradiol, resulted in significantly increased wet and blotted uterine weights compared to the vehicle control group.

Table 4. Uterine Weights

Dose Group	Dose Level (mg/kg/day)	n	Wet Uterine Weight Mean (mg) ± SD	Blotted Uterine Weight Mean (mg) ± SD
Vehicle Control	0	8	73.3 ± 11.7	67.8 ± 12.9
Homosalate	320	8	81.7 ± 16.2	75.8 ± 15.4
Homosalate	1000	8	68.7 ± 11.4	63.5 ± 11.4
Padimate-O	320	8	73.0 ± 9.3	68.1 ± 9.1
Padimate-O	1000	8	67.6 ± 9.0	62.8 ± 8.1
Avobenzone	320	8	76.5 ± 12.0	71.1 ± 11.2
Avobenzone	1000	6	69.8 ± 4.2	64.4 ± 5.0
Ensulizole	320	8	77.0 ± 13.3	71.5 ± 12.5
Ensulizole	1000	8	69.9 ± 13.0	64.9 ± 13.1
17α-Ethinyl estradiol	0.05	8	**307.0 ± 206.1**†	**193.1 ± 67.7**†

Abbreviations: SD - standard deviation

†17α-Ethinyl estradiol statistically significant compared to the vehicle control mean

5.4 Performance Criteria

Mean blotted uterine weight of animals administered corn oil was less than 0.04% of body weight indicating the study met the performance criteria (Appendix VI).

Uterine weight data from the baseline positive control test are located in Appendix IX. The assay was conducted using immature rats and administration of 17α-ethinyl estradiol via subcutaneous injection. ILS conducted this assay prior to the U.S. EPA finalizing and releasing the Uterotrophic Assay testing guideline (U.S. EPA, 2009) and the stated preferences of the ovariectomized rat model.

SUMMARY

Ovariectomized adult female rats were orally administered vehicle control (corn oil), 320 or 1000 mg/kg/day Avobenzone, Ensulizole, Homosalate, Padimate-O, or 17α-ethinyl estradiol (positive control) for 3 consecutive days and then euthanized. Body weights and clinical observations were performed daily. At termination, uteri were excised and wet and blotted weights recorded.

Following administration of 17α-ethinyl estradiol, wet and blotted uterine weights were significantly increased compared to vehicle controls indicating a positive response in the animal model.

Administration of Padimate-O and Avobenzone significantly decreased body weight gain at 1000 mg/kg/day, but not at 320 mg/kg/day compared to vehicle control animals. Administration of Homosalate significantly decreased final body weight at 1000 mg/kg/day, but not 320 mg/kg/day. Body weight gain was not significantly different at either dose level of Homosalate. Administration of Ensulizole did not affect body weights or body weight gain compared to vehicle controls.

Uterine weights (wet and blotted) did not significantly change at either dose level for any of the test substances compared to the vehicle control.

Oral administration of Avobenzone, Ensulizole, Homosalate, or Padimate-O up to the limit dose level of 1000 mg/kg/day, were not estrogenic in the ovariectomized rat model Uterotrophic Assay (OPPTS 890.1600).

REFERENCES

Aillon, K. (2012a). Dose Formulation Development Study of Avobenzone in Corn Oil. MRI Project Number-NTP ChemTask Number: 110730-Chem10987. Unpublished study report prepared by Midwest Research Institute.

Aillon, K. (2012b). Formulation Preparation and Analysis of Avobenzone in Corn Oil for ILS, Inc. MRI Project Number-NTP ChemTask Number: 110730-Chem11723. Unpublished study report prepared by Midwest Research Institute.

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Blake, J. (2012a). Ensulizole in Corn Oil Dose Formulation Development. RTI Project Number-ChemTask Number: 0212839.200.003.066-Chem11145. Unpublished study report prepared by Research Triangle Institute, International.

Blake, J. (2012b). Ensulizole in Corn Oil Formulation Analysis. RTI Project Number-ChemTask Number: 0212839.200.003.075-Chem11718. Unpublished study report prepared by Research Triangle Institute, International.

Blake, J. (2012c). Homosalate in Corn Oil Dose Formulation Development. RTI Project Number-ChemTask Number: 0212839.200.003.063-Chem11139. Unpublished study report prepared by Research Triangle Institute, International.

Blake, J. (2012d). Homosalate in Corn Oil Formulation Analysis. RTI Project Number-ChemTask Number: 0212839.200.003.077-Chem11720. Unpublished study report prepared by Research Triangle Institute, International.

Blake, J. (2012e). Padimate-O in Corn Oil Formulation Analysis. RTI Project Number-ChemTask Number: 0212839.200.003.076-Chem11719. Unpublished study report prepared by Research Triangle Institute, International.

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KEY PERSONNEL

Study Director:
Principal Toxicologist:
Toxicology Study Manager:
Animal Facility Operations Manager:
Necropsy Manager:
Facility Veterinarian:
Health and Safety Manager:
Dose Formulations:

Appendix I
Certificate of Analysis

N135-247 [redacted] 3-2212

From: [redacted]
To:
Subject: C of A for lot 7862K
Date: Tuesday, April 05, 2011 3:18:17 PM
Attachments: ATT00002.jpe

----- Forwarded by [redacted] on 04/05/2011 03:12 PM -----

MP Biomedicals, LLC
29525 Fountain Parkway
Solon, Ohio 44139
Telephone: 440/337-1200
Toll Free: 800/854-0530
Fax: 440/337-1180
web: www.mpbio.com

Certificate of Analysis

Product Description: Corn Oil
Catalog Number: 901414
Lot: 7862K

Formula: N/A
CAS #: 8001-30-7
Physical Description: Yellow Oil
Formula Weight: N/A
Storage: Room Temperature

Test	Specification	Result
Identity	Passes	Passes

Color (Lovibond): 1.6

UNIVERSAL PRESERV A-CHEM INC.

Chemicals for Food, Pharmaceutical, Cosmetic and Industrial Trades

N135-247 [redacted] 3-21-12

CERTIFICATE OF ANALYSIS

Product: AVOBENZONE USP

UPI Code # 830027
CAS # 70356-09-1
Lot # L802809

Manufacture Date: 12/31/2008
Recommended Retest Date: 12/31/2011

TEST	SPECIFICATIONS		TEST RESULTS
	MIN	MAX	
Description	Off White to Yellowish Crystalline Powder		Pass
Assay, %	95.00	100.00	98.30
Impurities	Not Detectable		None Detected
Identification A, B	Passes Tests		Conforms
Melting Range, °C	81.0	86.0	83.0
Loss on Drying, %		0.50	0.01
Extinction	1100.00	1210.00	1198.00

App: JB/PG/DM

Approved By: [redacted] Technical Director

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60 Jiffy Road * Somerset, NJ 08873-3438

PHONE:732 568-1266 FAX:732 568-1299 WEBSITE:UPICHEM.COM EMAIL: INFO@UPICHEM.COM

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Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.080
ChemTask No. CHEM11786
CAS No. 27503-81-7

This pdf is an exact duplicate of the original approved report

[redacted]
Program Information Coordinator

ENSULIZOLE

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted] 09-05-12
Task Leader Date

Approved by:

[redacted] 09/05/12
Reshan Fernando, Ph.D. Date
Principal Investigator

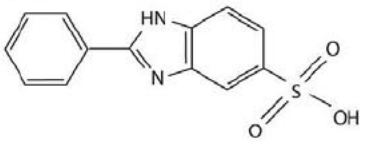
Submitted to:

[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

ENSULIZOLE

CAS No.: 27503-81-7	Study Lab: (Investigator): ILS [redacted]
RTI Chemical ID Code: N60	Lot No. (Vendor): 05117JE(Aldrich)
ChemTask No.: CHEM11786	Vendor Purity: 99.9% (by HPLC, Aldrich COA)
RTI Log Nos. (Amt. Received): Analytical: 082010-C-15 (~50 g) Reference: 082010-C-05 (~5 g)	Receipt Date: Aug 20, 2010 (Bulk receipt and reference)
Program Supported: TOX	Receipt Condition: No damage noted
Analysis Dates: May 11, 15 and 24, 2012	Submitter: [redacted]
Interim Results Date: May 29, 2012	Shipping Container: NA (in-house transfer)
	Storage Conditions: Bulk: Room temperature Reference: Freezer (~ -20 °C)

STRUCTURE



MOL. WT.
274.30

MOL. FORMULA
$C_{13}H_{10}N_2O_3S$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, an aliquot of ensulizole was submitted for bulk chemical reanalysis. Chemical purity of the bulk sample was determined relative to a reference standard of the same lot/batch number which had been stored at RTI under freezer conditions. Analytical results obtained by LC chromatographic method indicated that the sample had a percent relative purity of 99.6% when compared to the frozen reference standard. The FTIR spectrum of the bulk sample matched the spectrum of the frozen reference and was consistent with the structure for ensulizole.

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Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.082
ChemTask No. CHEM11788
CAS No. 118-56-9

This pdf is an exact duplicate of the original approved report.

[redacted]
Program Information Coordinator

HOMOSALATE

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted] 09-05-12
Date
Task Leader

Approved by:

[redacted] 09/05/12
Reshan Fernando, Ph.D. Date
Principal Investigator

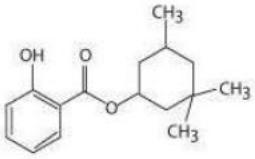
Submitted to:

[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

HOMOSALATE

CAS No.: 118-56-9	Study Lab: (Investigator): ILS [redacted]
RTI Chemical ID Code: N67	Lot No. (Vendor): YT0976 (Spectrum)
ChemTask No.: CHEM11788	Vendor Purity: 99.88% (Spectrum COA)
RTI Log Nos. (Amt. Received): Analytical: 091410-A-14 (~50 g) Reference: 091410-A-05 (~5 g)	Receipt Date: Sep 14, 2010 (Bulk) Receipt Condition: No damage noted
Program Supported: TOX	Submitter: [redacted]
Analysis Date: May 11, 21-23, 2012	Shipping Container: NA (in-house transfer)
Interim Results Date: May 29, 2012	Storage Conditions: Bulk: Room temperature Reference: Freezer (~-20 °C)

STRUCTURE



MOL. WT.
262.34

MOL. FORMULA
$C_{16}H_{22}O_3$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, an aliquot of homosalate was submitted for bulk chemical reanalysis. Chemical purity of the bulk sample was determined relative to a reference standard of the same lot/batch number which had been stored at RTI under freezer conditions. Analytical results obtained by a GC/FID chromatographic method indicated that the sample had a percent relative purity of 99.3% when compared to the frozen reference standard. The FTIR spectrum of the bulk sample matched the spectrum of the frozen reference and was consistent with an identity of homosalate.

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Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.081
ChemTask No. CHEM11787
CAS No. 21245-02-3

This pdf is an exact duplicate of the original approved report.

[redacted]
Program Information Coordinator

2-ETHYLHEXYL-P-DIMETHYL-AMINOBENZOATE (PADIMATE O)

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted] 09-05-12
Date
Task Leader

Approved by:

[redacted] 09/05/12
Date
Keshan Fernando, Ph.D.
Principal Investigator

Submitted to:

[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

2-ETHYLHEXYL-P-DIMETHYL-AMINOBENZOATE (PADIMATE O)

CAS No.: 21245-02-3

RTI Chemical ID Code: L98

ChemTask No.: CHEM11787

RTI Log Nos. (Amt. Received):
Bulk Analytical: 082010-B-14 (~50 g)
Reference: 082010-B-05 (~5 g)

Program Supported: TOX

Analysis Dates: May 21-22, 24, 2012

Interim Results Date: May 30, 2012

Study Lab: (Investigator): ILS [redacted]

Lot No. (Vendor): MKBF0590V (Aldrich)

Vendor Purity: 98.3% (Aldrich COA)

Receipt Date: Aug 20, 2010 (Bulk)

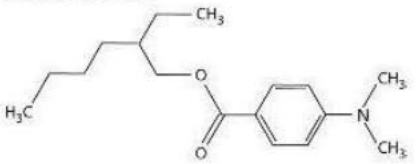
Bulk Receipt Condition: Good, room temperature

Submitter: [redacted]

Shipping Container: NA (in-house transfer)

Storage Conditions:
Bulk: Room temperature
Reference: Freezer (~-20 °C)

STRUCTURE



MOL. WT.
277.40

MOL. FORMULA
$C_{18}H_{27}NO_2$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, an aliquot of padimate O was submitted for bulk chemical reanalysis. Chemical purity of the bulk sample was determined relative to a reference standard of the same lot/batch number which had been stored at RTI under freezer conditions. Analytical results obtained by a GC/FID chromatographic method indicated that the sample had a percent relative purity of 98.1% when compared to the frozen reference standard. The FTIR spectrum of the bulk sample matched the spectrum of the frozen reference and was consistent with an identity of padimate O.

N135-247 [redacted] 3-22-12 Page 1 of 1

Certificate of Analysis

SIGMA-ALDRICH

Product Name	17α-Ethynylestradiol, ≥98%
Product Number	E4876
Product Brand	SIGMA
CAS Number	57-63-6
Molecular Formula	$C_{20}H_{24}O_2$
Molecular Weight	296.40

TEST	SPECIFICATION	LOT 090M1241V RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER	WHITE POWDER
SOLUBILITY	CLEAR COLORLESS TO FAINT YELLOW SOLUTION AT 50MG/ML IN ETHANOL	CLEAR COLORLESS
UV-VIS SPECTRUM	EMM = 2.01 TO 2.10 AT LAMBDA MAX 281 TO 282NM IN ETHANOL	EMM = 2.07 AT LAMBDA MAX 281NM
PURITY BY HPLC	MINIMUM 98%	99%
QC RELEASE DATE		SEP 2010
PRODUCT CROSS REFERENCE INFORMATION		REPLACEMENT FOR ALDRICH #285862

[redacted] Manager
Quality Control
St. Louis, Missouri USA

http://www.sigmaaldrich.com/catalog/CertOfAnalysisPage.do?symbol=E4876&LotNo=09... 3/20/2012

Appendix II:

Dose Formulation Analysis

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[redacted]
Program Information Coordinator

Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.075
ChemTask No. CHEM11718
CAS No. 27503-81-7

ENSULIZOLE

IN CORN OIL

FORMULATION ANALYSIS

Mix Dates: March 22 and April 3, 2012

July 13, 2012

Prepared by:

[redacted] 07/13/12
Date
Task Leader

Approved by:

[redacted] 07/13/12
Reshan Fernando, Ph.D. Date
Principal Investigator

Submitted to:

[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

ENSULIZOLE

CAS No.: 27503-81-7

ChemTask No.: CHEM11718

RTI Chemical ID Code: N60

Program Supported: TOX

Analysis Dates: Mar 27-28, Apr 3-5, 2012

Interim Results Dates: Mar 30, Apr 5, Apr 6, 2012

Mix Dates: Mar 22, Apr 3, 2012

Lot No. (Vendor): 05117JE (Aldrich)

Vendor Purity: 99.9% (Aldrich COA)

Vehicle: Corn oil

Vehicle Lot No.: (Vendor): 2AE0415 (Spectrum Chemical Mfg. Corp.)

Submitter: ILS

Study Lab (Investigator): ILS ([redacted])

Samples Received (RTI Log Nos.):

Mar 23 receipt: 9 x 30 mL, 032312-B-01 to -03 – 200 mg/mL; 032312-B-04 to -06 – 64 mg/mL; 032312-B-07 to -09 – 20 mg/mL; and 1 x 100 mL, 032312-B-10 – 0 mg/mL.

Apr 3 receipt: 6 x 30 mL, 040312-A-02 to -04 – 20 mg/mL; 040312-A-05 to -7 – 64 mg/mL; and 1 x 100 mL, 040312-A-01 - 0 mg/mL

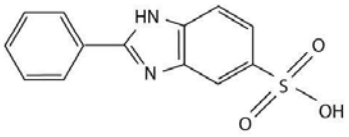
Dose Formulation Concentrations: 200 mg/mL; 64 mg/mL 20 mg/mL; 0 mg/mL

Sample Receipt Date: Mar 23, 2012 and Apr 3, 2012

Sample Containers: March 23 receipt: 9 amber glass bottles; 1 amber glass bottle April 3 receipt: 6 amber glass bottles; 1 amber glass bottle

Receipt Condition: Good

Storage Condition: Room temperature

STRUCTURE	MOL. WT.	MOL. FORMULA
	274.30	$C_{13}H_{10}N_2O_3S$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, a formulation analysis was performed to determine the ensulizole content and homogeneity of dose formulations and a vehicle blank prepared in corn oil, submitted by the study lab.

Upon initial analysis using a LC method, results for the three high dose samples showed a mean concentration of 189 mg/mL, which was 94.5% of the nominal concentration, with a relative standard deviation value of 10.3% (n=9). The middle and low dose samples showed

mean concentrations of 56.6 mg/mL (88.4% of nominal, 11.7% RSD) and 17.8 mg/mL (89.0% of nominal, 9.1% RSD), respectively. No test chemical was detected in the blank sample (limit of detection was 0.366 mg/mL). While no single determinate error was uncovered, the ILS Study Director elected to reformulate the low and middle dose formulations (20 and 64 mg/mL) and submit for reanalysis.

The low dose and middle dose concentrations were re-prepared at the study lab on April 3, 2012, and submitted to RTI for analysis.

The low dose samples (20 mg/mL) showed a mean concentration of 18.5 mg/mL (92.5% of nominal, 11.2% RSD). No test chemical was detected in the blank sample. The mid-level dose samples (64 mg/mL) again showed poor precision (RSD = 17.1%), with the bottom homogeneity sample showing the greatest variability. Therefore, a review of the analytical procedure was conducted but did not uncover any determinate errors. However, a slight modification to the pipetting technique was made to ensure consistent draw/dispensing of analytical aliquots for analysis. The bottom mid-level formulation was re-aliquotted and re-analyzed, and showed acceptable results with a mean concentration of 63.9 mg/mL (99.8% of nominal, 8.6% RSD).

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: **Ensulizole**

Task Type: **Formulation Analysis**

RTI Task Number: **0212839.200.003.075**

Chem Task Number: **CHEM11718**

This study/task was audited by the Regulatory and Quality Assurance (RQA) – Quality Assurance Unit and the results of the inspections and audits were reported to the task leader/study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Process Inspection - Formulation Analysis	03/27/2012	03/28/2012
Data and Report Audit	06/19-21/12	06/21/2012

Prepared by:

[redacted]
Quality Assurance Specialist

07/13/2012
Date

Reviewed by:

[redacted]
Quality Assurance Specialist

7/13/2012
Date

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TABLE OF CONTENTS

Figures

ENSULIZOLE

1.0 INTRODUCTION

The purpose of this work was to determine the ensulizole content and homogeneity of corn oil suspensions submitted by the study lab. To accomplish this, a formulation analysis was performed.

2.0 SAMPLE IDENTIFICATION

The following samples were received at RTI on March 23, 2012, for ensulizole analysis.

RTI Log No.	Target Conc. (mg/mL)	Sample ID	Dose Group (mg/kg)	Expiration Date
032312-B-01	200	12-25-1T	1000	May 4, 2012
032312-B-02	200	12-25-1M	1000	May 4, 2012
032312-B-03	200	12-25-1B	1000	May 4, 2012
032312-B-04	64	12-25-2T	320	May 4, 2012
032312-B-05	64	12-25-2M	320	May 4, 2012
032312-B-06	64	12-25-2B	320	May 4, 2012
032312-B-07	20	12-25-3T	100	May 4, 2012
032312-B-08	20	12-25-3M	100	May 4, 2012
032312-B-09	20	12-25-3B	100	May 4, 2012
032312-B-10	0	N135-11-121-32212	NA	May 4, 2012

The following reformulated samples were received at RTI on April 3, 2012, for analysis.

RTI Log No.	Target Conc. (mg/mL)	Sample ID	Dose Group (mg/kg)	Expiration Date
040312-A-01	0	N135-11-121-4312	NA	May 16, 2012
040312-A-02	20	12-41-1T	100	May 16, 2012
040312-A-03	20	12-41-1M	100	May 16, 2012
040312-A-04	20	12-41-1B	100	May 16, 2012
040312-A-05	64	12-41-2T	320	May 16, 2012
040312-A-06	64	12-41-2M	320	May 16, 2012
040312-A-07	64	12-41-2B	320	May 16, 2012

3.0 SAMPLE ANALYSIS

The methodology used for determining concentrations of ensulizole in the dose formulations is described in the RTI International report "Ensulizole in Corn Oil, Dose Formulation Development", (CHEM11145), January 11, 2012. A summary of the method is attached as an appendix to this report.

4.0 SAMPLE RESULTS

4.1 Mix Date March 22, 2012

The concentrations of ensulizole found in the dose formulations are tabulated below. Found concentrations are reported in units of mg/mL; percent recovery (versus the nominal concentration) was calculated using these values.

RTI Log No	Nominal Conc. (mg/mL) (Sampling Location)	Found Conc.[a] (mg/mL)	Mean Found Conc. (n=3) (mg/mL)	Mean Found/ Nominal	Mean Found Conc. (n=9) (mg/mL)	Mean Found/ Nominal
032312-B-01-1[b]	200 (top)	233	203 (13.6% RSD)	102%	189 (10.3% RSD)	94.5%
032312-B-01-2		180				
032312-B-01-3		196				
032312-B-02-1	200 (middle)	174	182 (8.1% RSD)	91.0%		
032312-B-02-2		173				
032312-B-02-3		199				
032312-B-03-1	200 (bottom)	191	181 (4.8% RSD)	90.5%		
032312-B-03-2		174				
032312-B-03-3		179				
032312-B-04-1[b]	64 (top)	60.9	52.8 (13.9% RSD)	82.5%	56.6 (11.7% RSD)	88.4%
032312-B-04-2		46.6				
032312-B-04-3		50.9				
032312-B-05-1	64 (middle)	61.9	61.3 (12.0% RSD)	95.8%		
032312-B-05-2		53.7				
032312-B-05-3		68.4				
032312-B-06-1	64 (bottom)	52.4	55.6 (5.5% RSD)	86.9%		
032312-B-06-2		55.9				
032312-B-06-3		58.5				
032312-B-07-1[b]	20 (top)	18.1	17.6 (7.1% RSD)	88.0%	17.8 (9.1% RSD)	89.0%
032312-B-07-2		16.1				
032312-B-07-3		18.5				
032312-B-08-1	20 (middle)	17.8	19.1 (6.1% RSD)	95.5%		
032312-B-08-2		20.0				
032312-B-08-3		19.4				
032312-B-09-1	20 (bottom)	14.7	16.9 (11.6% RSD)	84.5%		
032312-B-09-2		17.6				
032312-B-09-3		18.4				
032312-B-10-1[b]	0 NA	ND[c]	NA	NA	NA	NA
032312-B-10-2		ND				
032312-B-10-3		ND				

[a]Quantitation based on the weighted (1/x) linear regression equation: y = 0.03005x + 0.007889; r = 0.9993.
[b]The numerical suffix (1, 2, 3) indicates the respective analytical aliquot for the given sample.
[c]ND=Not detected; limit of detection (LOD) = 0.366 mg/mL; limit of quantitation (LOQ) = 1.22 mg/mL.

Quality control samples prepared and analyzed with the dose formulations gave acceptable relative errors (3.7% and 9.0%), indicating acceptable analytical control. Based on the data reported in the table above and standard acceptance criteria, the low and middle mixes are not acceptable, showing poor precision (at least one of the dose formulation samples exhibited >10% RSD, and for the middle dose concentration the overall precision was >10%) and poor

accuracy (both < 90% of nominal). A second formulation for the low and middle doses was prepared on April 3, 2012 and samples submitted for dose formulation analysis.

4.2 Mix Date April 3, 2012

The concentrations of ensulizole found in the dose formulations are tabulated below. Found concentrations are reported in units of mg/mL; percent recovery (versus the nominal concentration) was calculated using these values.

RTI Log No	Nominal Conc. (mg/mL) (Sampling Location)	Found Conc.[a] (mg/mL)	Mean Found Conc. (n=3) (mg/mL)	Mean Found/ Nominal	Mean Found Conc. (n=9) (mg/mL)	Mean Found/ Nominal
040312-A-05-1[b]	64 (top)	65.9	67.4 (3.7% RSD)	105%		
040312-A-05-2		66.1				
040312-A-05-3		70.3				
040312-A-06-1	64 (middle)	60.2	57.3 (5.2% RSD)	89.5%	59.3 (17.1% RSD)	92.7%
040312-A-06-2		54.3				
040312-A-06-3		57.3				
040312-A-07-1	64 (bottom)	70.7	53.1 (28.8% RSD)	83.0%		
040312-A-07-2		42.9				
040312-A-07-3		45.8				
040312-A-02-1[b]	20 (top)	22.0	19.5 (13.7% RSD)	97.5%		
040312-A-02-2		16.7				
040312-A-02-3		19.7				
040312-A-03-1	20 (middle)	16.2	17.5 (12.0% RSD)	87.5%	18.5 (11.2% RSD)	92.5%
040312-A-03-2		19.9				
040312-A-03-3		16.4				
040312-A-04-1	20 (bottom)	18.8	18.6 (8.7% RSD)	93.0%		
040312-A-04-2		16.9				
040312-A-04-3		20.1				
040312-A-01-1[b]	0 NA	ND[c]	NA	NA	NA	NA
040312-A-01-2		ND				
040312-A-01-3		ND				

[a]Quantitation based on the weighted (1/x) linear regression equation: y = 0.02847x + 0.01060; r = 0.9991.

[b]The numerical suffix (1, 2, 3) indicates the respective analytical aliquot for the given sample.

[c]ND=Not detected; limit of detection (LOD) = 0.366 mg/mL; limit of quantitation (LOQ) = 1.22 mg/mL.

Quality control samples prepared and analyzed with the dose formulations gave acceptable relative errors (3.8% and 0.2%), indicating acceptable analytical control.

The mid-level dose formulation samples showed poor precision (RSD = 17.1%), with the bottom homogeneity sample showing the greatest variability. The bottom mid-level formulation

was re-aliquotted and re-analyzed using the same system and standards as the analysis reported in this section.

4.3 Mix Date April 3, 2012: Re-Preparation and Analysis

The concentrations of ensulizole found in the re-prepared mid-level dose formulation are tabulated below. Found concentrations are reported in units of mg/mL; percent recovery (versus the nominal concentration) was calculated using these values.

RTI Log No	Nominal Conc. (mg/mL) (Sampling Location)	Found Conc.[a] (mg/mL)	Mean Found Conc. (n=3) (mg/mL)	Mean Found/ Nominal	Mean Found Conc. (n=9) (mg/mL)	Mean Found/ Nominal
040312-A-05-1[b]	64 (top)	65.9[c]	67.4 (3.7% RSD)	105%		
040312-A-05-2		66.1[c]				
040312-A-05-3		70.3[c]				
040312-A-06-1	64 (middle)	60.2[c]	57.3 (5.2% RSD)	89.5%	63.9 (8.6% RSD)	99.8%
040312-A-06-2		54.3[c]				
040312-A-06-3		57.3[c]				
040312-A-07-7	64 (bottom)	64.1[d]	67.1 (4.1% RSD)	105%		
040312-A-07-8		67.5[d]				
040312-A-07-9		69.6[d]				

[a]Quantitation based on the weighted (1/x) linear regression equation: y = 0.02847x + 0.01060; r = 0.9991.
[b]The numerical suffix (1, 2, 3) indicates the respective analytical aliquot for the given sample.
[c]Result from analysis on 4/4/12 detailed in Section 4.2.
[d]Re-aliquotted and analyzed on 4/5/12.

Quality control samples prepared and analyzed with the dose formulations gave acceptable relative errors (5.1% and 0.9%), indicating acceptable analytical control. The mid-level dose formulation samples showed acceptable results with a mean concentration of 63.9 mg/mL (99.8% of nominal, 8.6% RSD).

4.4 Summary

Representative chromatograms are shown in Figure 1. The vehicle standards plot is illustrated in Figure 2 for the weighted (1/x) linear regression equation y = 0.03005x + 0.007889, r=0.9993.

The RTI analytical laboratory provided the study lab (ILS) with sample analysis and results so the ILS Study Director could make prompt decisions regarding reformulation of any samples that appeared to deviate from normal acceptance criteria (±10 % of nominal, ≤10% RSD). In addition, RTI provided guidance on preparing formulations and reviewed the analytical procedure to ensure a high degree of accuracy and precision for the results. While no single determinate cause was uncovered that may have contributed significantly to the unexpected results, the following suggestions and/or modifications were incorporated.

1) Smaller batch sizes of study formulations than previously tested (< 1L) may have contributed to unexpected results, and the current acceptance criteria may not be appropriate for the smaller size.

2) Bulk formulations required additional mixing time due to propensity of test chemical to disperse and settle before handling (sampling or aliquotting).

3) Slight modifications to drawing/dispensing of analytical aliquots were incorporated to ensure consistent aliquotting for analysis.

The NTP COTR and ILS Study Director were informed of the results and elected to continue with the study.

5.0 ACKNOWLEDGMENT

Personnel contributing to the performance of this task included: [redacted]

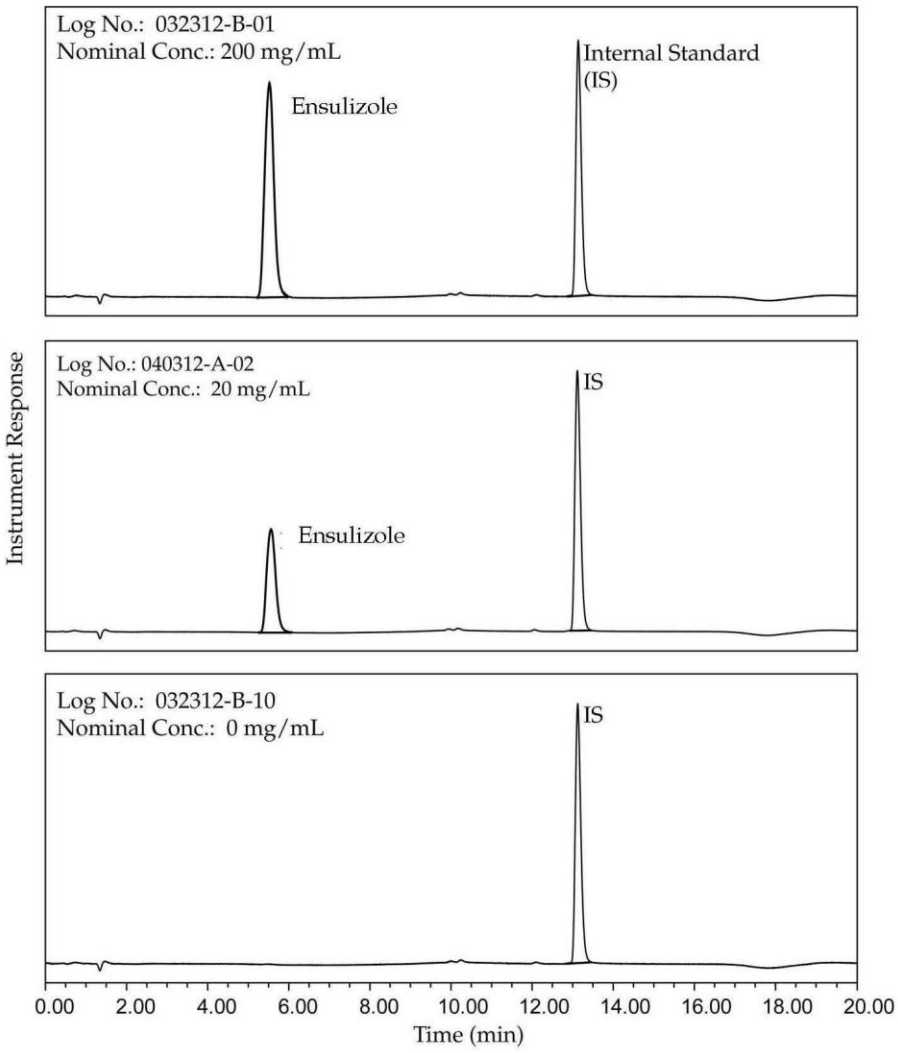


Figure 1. Representative Liquid Chromatograms of Ensulizole in Corn Oil

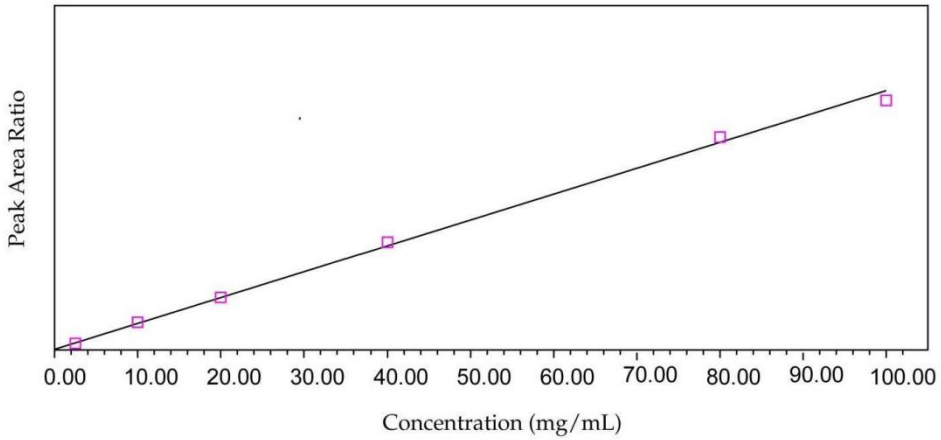


Figure 2. Plot of Vehicle Standards Data – Ensulizole in Corn Oil

APPENDIX

Method Summary

Determination of Ensulizole in Corn Oil

This appendix summarizes the method used to prepare formulation samples of ensulizole in corn oil for analysis, and describes the liquid chromatography (LC) method.

Preparation of the Internal Standard

An internal standard (IS) stock solution was prepared by transferring ~500 mg of padimate O into a 10-mL volumetric flask and diluting to volume with mobile phase B, then mixing by inversion. The IS stock (50 mg/mL) was mixed by inversion.

A working IS solution (WIS) was prepared by transferring 1.0 mL of the IS stock solution to a 1-L volumetric flask, diluting to volume with mobile phase B, and then mixing by inversion. The working IS solution (0.05 mg/mL) was transferred to a bottle for ambient storage.

Preparation of Vehicle Stock Standards

Two vehicle stock standards (VA and VB) were prepared by weighing out aliquots of ensulizole and dissolving them in 50 mL of the corn oil vehicle.

VA was prepared with ~6.25 g of ensulizole and had a final concentration of 125 mg/mL. VB was prepared from ~ 5.0 g of ensulizole and had a final concentration of 100 mg/mL.

Preparation of Vehicle and QC Standards

The standards were prepared by diluting the spiking solutions in corn oil vehicle as described in the table below. The vehicle standards were mixed briefly by vortex action, sonicated for ~20 minutes, and stirred magnetically until homogeneous (~30 minutes) and during sampling. Two additional vehicle standards were prepared as quality control (QC) standards at the VB1 and VA3 concentrations.

Vehicle Standards

Vehicle Std ID	Spiking Solution	Spike Volume (mL)	Final Volume (mL)	Nominal Vehicle Std Conc. (mg/mL)	Actual Vehicle Std Conc.[a] (mg/mL)
VA1	VA	8.0	10	100	100
VB1	VB	8.0	10	80.0	80.0
VA2	VA	3.2	10	40.0	40.0
VB2	VB	2.0	10	20.0	20.0
VA3	VA	1.6	20	10.0	10.0
VB3	VB	0.5	20	2.50	2.50

[a]Example Calculation, VA1: 125 mg/mL x 8.0 mL/10 mL = 100 mg/mL.

Preparation of Formulation Samples for Analysis

All formulation samples were mixed briefly by vortex action, sonicated for ~20 minutes and then stirred on a magnetic stir plate for 3-4 hours. Dose formulations with concentrations between 80 and 340 mg/mL were diluted (1 mL to 5 mL) with corn oil, mixed briefly by vortex action, sonicated for ~ 20 minutes, stirred on a magnetic stir plate for ~30 minutes, and then prepared for analysis. All samples were prepared in triplicate for analysis.

Each sample, diluted sample, vehicle standard, vehicle blank, solvent blank (prepared from mobile phase B) and QC standard was prepared for analysis by transferring 0.050 mL to a scintillation vial and adding 20 mL of WIS. The vials are mixed by vortex action, sonicated for ~ 10 minutes, then centrifuged for ~ 10 minutes. A 1.5-mL aliquot of the supernatant is transferred to a 2-mL centrifuge tube and 0.5 mL of mobile phase A is added. The tube is mixed by vortex action, then centrifuged for ~ 5 minutes. An aliquot is transferred to an autosampler vial for analysis.

LC Analysis

Instrument	Waters Alliance 2695
Column	Waters XBridge C18, 3.5 µm particle size, 2.1 x 100 mm. 40 °C
Data System	Empower 2; Build 2154
Mobile Phases	A: Deionized water with 0.1% formic acid B: Methanol with 0.1% formic acid
Gradient Program	10% B for 0.67 min., ramp to 65% B in 4.33 min., ramp to 90% B in 1 min., hold for 7 min. Reverse to 10% B in 2 min., hold for 5 min.
Flow rate	0.25 mL/min.
Injection Volume	3 µL
Detector: Gas flows	Waters PDA 2996, 312 nm

For each dose formulation, a peak area ratio was calculated (sample area ÷ IS peak area). The found concentration of the analyte was calculated using the peak area ratios and the linear regression equations (weighted 1/x), applying a dilution factor as necessary. A mean found concentration was determined for each sampling location (n=3), and for overall homogeneity confirmation of each formulation (n=9).

Acceptance criteria for each formulation were a final found concentrations within +/- 10% of the nominal concentration, and a precision (expressed as relative standard deviation for the triplicate preparations) of ≤ 10%. Acceptance criteria for analysis was a regression line with r≥0.99, and QC standards with recovery ≤10% of the nominal concentration.

Analytical Chemistry Services for the NTP
NIEHS Contract No.: HHSN273201100001C
MRI Project No.: 110730
NTP ChemTask No.: CHEM11723

Formulation Preparation and Analysis Final Report

Avobenzone

Formulation Preparation and Analysis of Avobenzone in Corn Oil for ILS, Inc.

MRI Assignment No.: 2236

July 17, 2012

Submitted by:

MRIGlobal
425 Volker Boulevard
Kansas City, MO 64110-2241

Submitted to:

[redacted]
National Institute of Environmental Health Sciences
111 T. W. Alexander Drive, MD K2-07
P.O. Box 12233
Research Triangle Park, NC 27709-2233

Formulation Preparation and Analysis of Avobenzone in Corn Oil for ILS, Inc.

Analytical Chemistry Services for the NTP
NIEHS Contract No.: HHSN273201100001C
MRI Project No.: 110730
NTP ChemTask No.: CHEM11723
MRI Assignment No.: 2236
July 17, 2012

This PDF File is an Exact Copy of the Report
Signature: [redacted]
Date: 7-18-12

Prepared by:

[redacted]
Study Director

Approved by:

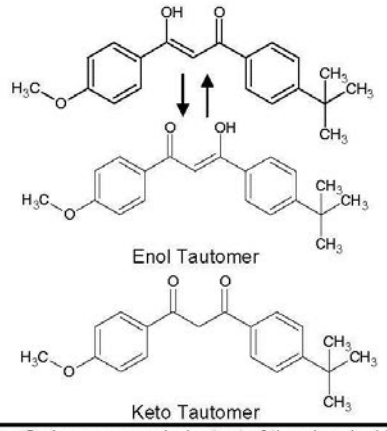
[redacted]
Joseph W. Algaier, Ph.D.
Principal Investigator

Reviewed by:

[redacted]
Group Leader

Formulation Preparation and Analysis of Avobenzone in Corn Oil for ILS, Inc.

Chemical Information: Avobenzone

CAS No.: 70356-09-1 MRI Assignment No: 2236 NTP ChemTask No.: CHEM11723 Program Supported: TOX Analysis Dates: 3/27/12, 3/29/12, and 3/30/12 Interim Report Dates: 3/29/12 and 3/30/12	Supplier: Universal Preserv-A-Chem, Inc. Lot No.: L802809 MRI Assigned Batch No.: 01 Purity: 98.30% (per CoA)[a] Appearance: Off white to yellowish crystalline powder (per CoA). Appearance as stated in the CoA was confirmed by visual observation Storage Condition @ MRI: Ambient; protected from light Vehicle: Corn Oil Vehicle Lot No.: ZT1301 (Spectrum Chemical Mfg. Corp.) Formulation Prepared: ~ 64 mg/mL Preparation Date: 3/30/12
Structure  	Molecular Weight: 310.39 Molecular Formula: $C_{20}H_{22}O_3$

[a] The CoA recommended retest of the chemical by December 31, 2011. The chemical was retested in the associated chemical comprehensive analysis, CHEM10985.[1]

[1] MRI Report, "Chemical Comprehensive Analysis Final Report, Chemical Comprehensive Analysis of Avobenzone," NIEHS Contract No. HHSN273201100001C, NTP ChemTask No. CHEM10985, MRI Project No. 110730, MRI Assignment No. 2003, February 16, 2012.

Formulations Prepared by ILS, Inc. for Analysis at MRI—Avobenzone in Corn Oil

MRI Assignment No.: 2236 NTP ChemTask No.: CHEM11723 ILS Protocol No.: N135-247, N135-248		Supplier: ILS, Inc. MRI Receipt Date: 3/23/12 Storage Conditions at MRI: Ambient Formulation Preparation Date: 3/22/12
ILS, Inc. sample ID	Formulation concentration (~ mg/mL)	MRI analysis sample name
N135-11-121-32212	0	N135-11-121-32212$_1$, N135-11-121-32212$_2$, N135-11-121-32212$_3$
12-19-1T	200	12-19-1T$_1$, 12-19-1T$_2$, 12-19-1T$_3$
12-19-1M	200	12-19-1M$_1$, 12-19-1M$_2$, 12-19-1M$_3$
12-19-1B	200	12-19-1B$_1$, 12-19-1B$_2$, 12-19-1B$_3$
12-19-2T	64	12-19-2T$_1$, 12-19-2T$_2$, 12-19-2T$_3$
12-19-2M	64	12-19-2M$_1$, 12-19-2M$_2$, 12-19-2M$_3$
12-19-2B	64	12-19-2B$_1$, 12-19-2B$_2$, 12-19-2B$_3$
12-19-3T	20	12-19-3T$_1$, 12-19-3T$_2$, 12-19-3T$_3$
12-19-3M	20	12-19-3M$_1$, 12-19-3M$_2$, 12-19-3M$_3$
12-19-3B	20	12-19-3B$_1$, 12-19-3B$_2$, 12-19-3B$_3$

Executive Summary

The purpose of this study was to analyze formulations of avobenzone in corn oil at ~ 0, ~ 20, ~ 64, and ~ 200 mg/mL prepared by ILS, Inc. (ILS, Inc., Protocol No. N135-248, N135-247). The formulations were analyzed using a validated high performance liquid chromatography with ultraviolet detection (HPLC/UV) method. In addition, an ~ 64 mg/mL formulation was prepared and analyzed for formulation concentration and homogeneity at Midwest Research Institute (MRI) for shipment to ILS, Inc., under Shipment Assignment CHEM11733.[2]

Formulation samples received from ILS, Inc., at ~ 0, ~ 20, ~ 64, and ~ 200 mg/mL were analyzed for avobenzone concentration and all formulations were within criteria (< 10% of target) as shown in the table below.

ILS, Inc. sample name	MRI sample name	Target formulation concentration (mg/mL)	Average % of target
N135-11-121-32212	N135-11-121-32212$_1$ N135-11-121-32212$_2$ N135-11-121-32212$_3$	0	NA
12-19-1T	12-19-1T$_1$, 12-19-1T$_2$, 12-19-1T$_3$		104.5 ± 0.5 (s)
12-19-1M	12-19-1M$_1$, 12-19-1M$_2$, 12-19-1M$_3$	200	105.4 ± 0.8 (s)
12-19-1B	12-19-1B$_1$, 12-19-1B$_2$, 12-19-1B$_3$		104.4 ± 1.2 (s)
12-19-2T	12-19-2T$_1$, 12-19-2T$_2$, 12-19-2T$_3$		98.5 ± 0.9 (s)
12-19-2M	12-19-2M$_1$, 12-19-2M$_2$, 12-19-2M$_3$	64	97.3 ± 0.4 (s)
12-19-2B	12-19-2B$_1$, 12-19-2B$_2$, 12-19-2B$_3$		97.2 ± 0.3 (s)
12-19-3T	12-19-3T$_1$, 12-19-3T$_2$, 12-19-3T$_3$		102.7 ± 0.8 (s)
12-19-3M	12-19-3M$_1$, 12-19-3M$_2$, 12-19-3M$_3$	20	102.6 ± 1.1 (s)
12-19-3B	12-19-3B$_1$, 12-19-3B$_2$, 12-19-3B$_3$		105.0 ± 3.8 (s)

During analysis of the ~ 64 mg/mL formulation, the preliminary results showed the formulation was ~ 32 mg/mL. To further evaluate, the ~ 64 mg/mL formulation was re-analyzed using a different dilution scheme to confirm the sample preparation method. After re-analysis, it was determined that the amount of IS was doubled in each sample. The concentration calculation was corrected by dividing the IS peak area by two before the PAR was calculated. With the correction factor applied, the formulation met criteria with percent of target values between 98.3% and 99.9% (see table below). The data with the correction factor applied is shown table above and on the following page.

ILS, Inc. sample name	MRI sample name	Target formulation concentration (mg/mL)	Average % of target
12-19-2T	R-12-19-2T$_1$, R-12-19-2T$_2$, R-12-19-2T$_3$		99.9 ± 0.7 (s)
12-19-2M	R-12-19-2M$_1$, R-12-19-2M$_2$, R-12-19-2M$_3$	64	98.3 ± 0.6 (s)
12-19-2B	R-12-19-2B$_1$, R-12-19-2B$_2$, R-12-19-2B$_3$		98.6 ± 0.8 (s)

[2] MRI Report, "Shipment Final Report, Shipment of Avobenzone and Avobenzone in Corn Oil Formulation to ILS, Inc.," NIEHS Contract No. HHSN273201100001C, NTP ChemTask No. CHEM11733, MRI Project No. 110730, MRI Assignment No. 2237, June 7, 2012.

To confirm the ~ 64 mg/mL formulation preparation method, MRI prepared a formulation of avobenzone in corn oil at ~ 64 mg/mL and analyzed the formulation for avobenzone concentration. The formulation was within criteria with percent of target values show in the table below.

MRI sample name	Formulation sampling location	Target formulation concentration (mg/mL)	Average % of target
64-T_1, 64-T_2, 64-T_3	Top	64	93.6 ± 0.4 (s)
64-M_1, 64-M_2, 64-M_3	Middle	64	92.8 ± 0.9 (s)
64-B_1, 64-B_2, 64-B_3	Bottom	64	91.4 ± 2.3 (s)

Quality Assurance Statement

Formulation Preparation and Analysis of Avobenzone in Corn Oil for ILS, Inc.

NTP ChemTask No.: CHEM11723
MRI Project No.: 110730
MRI Assignment No.: 2236

The Quality Assurance Unit (QAU) of MRI inspected this study and the findings were reported to the Study Director and Management as follows:

Phase inspected	Date inspected	Date reported
Protocol Audit	3/27/12	3/28/12
In-life Audit	3/27/12	3/28/12
Protocol Amendment No. 1 Audit	5/29/12	5/29/12
Protocol Amendment No. 2 Audit	5/29/12	5/29/12
Data Audit	5/29/12	5/29/12
Report Audit	5/29/12	5/29/12

In addition to the study specific audits/inspection cited above, inspection of applicable facilities and equipment was performed by the QAU and reports were submitted to management as follows:

Facility equipment	Date inspected	Date reported
LC Facility	1/31/12	1/31/12
285N Laboratory Complex	2/3/12	2/6/12

MIDWEST RESEARCH INSTITUTE

[redacted]
Senior Quality Assurance Officer

Approved:

[redacted]
Director, Quality and Regulatory Systems

July 17, 2012

Good Laboratory Practice Compliance Statement

Formulation Preparation and Analysis of Avobenzone in Corn Oil for ILS, Inc.

NTP ChemTask No.: CHEM11723
MRI Project No.: 110730
MRI Assignment No.: 2236

This study was conducted in compliance with the Good Laboratory Practice regulations of the U.S. Food and Drug Administration (21 *CFR* Part 58). The raw data and report will be stored in the MRI Archives.

[redacted]
Study Director

7/17/12
Date

Contents

Tables

Figures

Formulation Preparation and Analysis of Avobenzone in Corn Oil for ILS, Inc.

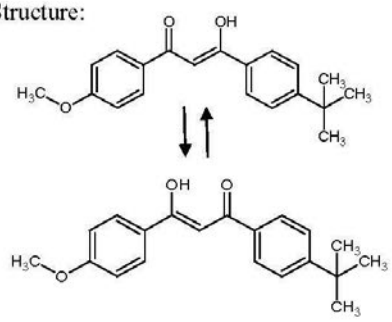
1. Introduction

The purpose of this study was to analyze formulations of avobenzone in corn oil at ~ 0, ~ 20, ~ 64, and ~ 200 mg/mL that were prepared at ILS, Inc. (ILS, Inc., Protocol No. N135-248, N135-247). The formulations were analyzed using a validated high performance liquid chromatography with ultraviolet detection (HPLC/UV) method. Additionally, a ~ 64 mg/mL formulation was prepared and analyzed for formulation concentration and homogeneity at Midwest Research Institute (MRI) on March 30, 2012. This formulation was shipped to ILS, Inc., under Shipment (SHIP) assignment CHEM11733.[3] This study was initiated on March 26, 2012.

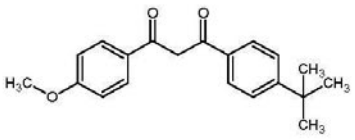
2. Chemical Information

Test Article:	Avobenzone
Supplier:	Universal Preserv-A-Chem, Inc.
Lot No.:	L802809
MRI-Assigned Batch No.:	01
Purity:	98.30% (per CoA)[4]
CAS No.:	70356-09-1
Molecular Formula:	$C_{20}H_{22}O_3$
Molecular Weight:	310.39

Structure:



Enol Tautomer



Keto Tautomer

[3] MRI Report, "Shipment Final Report, Shipment of Avobenzone and Avobenzone in Corn Oil Formulation to ILS, Inc.," NIEHS Contract No. HHSN273201100001C, NTP ChemTask No. CHEM11733, MRI Project No. 110730, MRI Assignment No. 2237, to be submitted.

[4] The CoA recommended retest of the chemical by December 31, 2011. The chemical was retested in the associated Chemical Comprehensive Analysis assignment. MRI Report, "Chemical Comprehensive Analysis Final Report, Chemical Comprehensive Analysis of Avobenzone," NIEHS Contract No. HHSN273201100001C, NTP ChemTask No. CHEM10985, MRI Project No. 110730, MRI Assignment No. 2003, February 16, 2012.

2.1 Formulations Prepared at ILS, Inc., and Analyzed at MRI

Formulations: Avobenzone in Corn Oil
Supplier: ILS, Inc.
ILS, Inc. Protocol No.: N135-248, N135-247
MRI Receipt Date: March 23, 2012

Table 1. Avobenzone in Corn Oil Formulations Prepared at ILS, Inc., and Analyzed at MRI

ILS, Inc., sample ID	Formulation concentration (~ mg/mL)	MRI analysis sample name
N135-11-121-32212	0	N135-11-121-32212$_1$, N135-11-121-32212$_2$, N135-11-121-32212$_3$
12-19-1T	200	12-19-1T$_1$, 12-19-1T$_2$, 12-19-1T$_3$
12-19-1M	200	12-19-1M$_1$, 12-19-1M$_2$, 12-19-1M$_3$
12-19-1B	200	12-19-1B$_1$, 12-19-1B$_2$, 12-19-1B$_3$
12-19-2T	64	12-19-2T$_1$, 12-19-2T$_2$, 12-19-2T$_3$
12-19-2M	64	12-19-2M$_1$, 12-19-2M$_2$, 12-19-2M$_3$
12-19-2B	64	12-19-2B$_1$, 12-19-2B$_2$, 12-19-2B$_3$
12-19-3T	20	12-19-3T$_1$, 12-19-3T$_2$, 12-19-3T$_3$
12-19-3M	20	12-19-3M$_1$, 12-19-3M$_2$, 12-19-3M$_3$
12-19-3B	20	12-19-3B$_1$, 12-19-3B$_2$, 12-19-3B$_3$

3. Materials and Equipment

High Performance Liquid Chromatography with Ultraviolet Detection System:

Waters 2695 Separations Module with a Waters 2487 Dual Absorbance Detector
TotalChrom Version 6.3.0 with a PE NCI902 Interface
Column, Waters, XTerra RP-18 (250 × 4.6 mm; 5 µm)
n-Decanophenone, Acros Organics, 99% purity, used as an internal standard
Corn Oil, Spectrum Chemical, NF Grade
Acetone, Burdick & Jackson, High Purity
Magnetic stir plates
Homogenizer, Polytron, PT-2100
Homogenizer, Polytron, PT-1200
Balance, Mettler Toledo, XS204
Balance, Mettler Toledo, XS205DU
PTFE Filters, Pall Gelman, Acrodisc, 0.45 µm
Syringes, BD, 3-mL, disposable

LC Autosampler Vials with screw cap lids
Volumetric glassware, Class A, low actinic or covered in aluminum foil, as needed

4. Analysis of ILS, Inc., Prepared Formulations

Formulations of avobenzone in corn oil prepared at ILS, Inc., at ~ 0, ~ 20, ~ 64, and ~ 200 mg/mL were analyzed using an HPLC/UV method validated from 3.8332 to 50.855 mg/mL under Dose Formulation Development (DFD) study, CHEM10987.[5] Formulations above ~ 50 mg/mL were diluted into the validated range.

4.1 Standards Preparation

4.1.1 Internal Standard (IS) Solution

An internal standard solution was prepared by accurately weighing and transferring ~ 1,000 mg of n-decanophenone into a 100-mL volumetric flask. The contents of the flask were diluted to volume with acetone and mixed by inversion for an expected concentration of ~ 10 mg/mL.

4.1.2 Stock Solutions

Two stock solutions (Stocks A and B) were prepared by accurately weighing and transferring 549.38 mg (Stock A) and 418.20 mg (Stock B) into individual 50-mL volumetric flasks. The contents of each flask were diluted to volume with acetone and mixed by inversion for expected concentrations of 10.988 mg/mL (Stock A) and 8.3640 mg/mL (Stock B).

4.1.3 Intermediate Solutions

Intermediate solutions (IB_1 to IA_6) were prepared by transferring aliquots from alternating stock solutions (Stocks A and B) into individual volumetric flasks. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 2).

NOTE: Stock B was used as IB_5 and Stock A was used as IA_6.

[5] MRI Report, "Dose Formulation Development Final Report, Dose Formulation Development Study of Avobenzone in Corn Oil", NIEHS Contract No. HHSN273201100001C, NTP ChemTask No. CHEM10987, MRI Project No. 110730, MRI Assignment No. 2005, February 17, 2012.

Table 2. Intermediate Solutions Preparation for ILS, Inc., Prepared Formulation Analysis

Intermediate solution	Stock solution	Stock solution aliquot (mL)	Volume (mL)	Expected analytical concentration (µg/mL)
IB_1	B	5	50	836.40
IA_2	A	10	50	2,197.5
IB_3	B	10	25	3,345.6
IA_4	A	10	25	4,395.0
IB_5	B	NA	NA	8,364.0
IA_6	A	NA	NA	10,988

4.1.4 Spiked Matrix Standards

Spiked matrix standards (B_{11} to A_{61}) were prepared by transferring 5 mL aliquots of the intermediate solutions (see Section 4.1.3) into individual 50-mL volumetric flasks each containing an ~ 1 g portion of corn oil and 2 mL of IS solution. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 3).

Table 3. Spiked Matrix Standards Preparation for ILS, Inc., Prepared Formulation Analysis

Spiked matrix standard	Intermediate solution	Intermediate solution aliquot (mL)	Matrix added (~ g)	Final volume (mL)[a]	Expected analytical concentration (µg/mL)	Expected formulation concentration (mg/mL)[b]
B_{11}	IB_1	5	1	50	83.640	3.8591
A_{21}	IA_2	5	1	50	219.75	10.139
B_{31}	IB_3	5	1	50	334.56	15.436
A_{41}	IA_4	5	1	50	439.50	20.278
B_{51}	IB_5	5	1	50	836.40	38.591
A_{61}	IA_6	5	1	50	1,098.8	50.696

[a] Contained 2 mL of IS solution.
[b] Density of matrix = 0.92278 g/mL (determined in the associated DFD, CHEM10987[3]).

4.2 Blanks Preparation

4.2.1 Reagent Blank

Acetone was used as the reagent blank (D_0).

4.2.2 IS Blank

An IS blank was prepared by transferring 2 mL of IS solution (Section 4.1.1) into a 50-mL volumetric flask. The contents of the flask were diluted to volume with acetone and mixed by inversion.

4.2.3 Matrix Blanks

A matrix blank (C_{01}) was prepared by transferring a ~ 1 g aliquot of corn oil into a 50-mL volumetric flask. The contents of the flask were diluted to volume with acetone and mixed by inversion.

A matrix blank with IS (C_{02}) was prepared by transferring a ~ 1 g aliquot of corn oil into a 50-mL volumetric flask containing 2 mL of IS solution. The contents of the flask were diluted to volume with acetone and mixed by inversion.

4.3 Formulation Sample Preparation

4.3.1 ~ 0 mg/mL Formulation

A stir bar was added to the container of the ~ 0 mg/mL formulation (N135-11-121-32212) and the container was placed on a stir plate. While stirring, triplicate aliquots (~ 1 g; accurately weighed) from the sample were transferred into individual 50-mL volumetric flasks each containing 2 mL of IS solution. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 4).

Table 4. Sample Preparation of ~ 0 mg/mL Formulation Prepared at ILS, Inc.

Formulation sample	Formulation aliquot (g)	Final volume (mL)[a]	Target concentration (mg/mL)
N135-11-121-32212$_1$	1.0144	50	0
N135-11-121-32212$_2$	1.0187	50	0
N135-11-121-32212$_3$	1.0153	50	0

[a] Contained 2 mL of IS solution.

4.3.2 ~ 20 mg/mL Formulation

The containers of the ~ 20 mg/mL formulation (12-19-3T, 12-19-3M, and 12-19-3B) were mixed using stir bars and magnetic stir plates. While stirring, triplicate aliquots (~ 1 g; accurately weighed) from each sample were transferred into individual 50-mL volumetric flasks each containing 2 mL of IS solution. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 5).

Table 5. Sample Preparation of ~ 20 mg/mL Formulation Prepared at ILS, Inc.

Formulation sample	Formulation container	Formulation aliquot (g)	Final volume (mL)[a]	Target concentration (mg/mL)
12-19-3T$_1$	12-19-3T	1.0422	50	20
12-19-3T$_2$	12-19-3T	0.9995	50	20
12-19-3T$_3$	12-19-3T	1.0107	50	20
12-19-3M$_1$	12-19-3M	1.0157	50	20
12-19-3M$_2$	12-19-3M	1.1190	50	20
12-19-3M$_3$	12-19-3M	1.0129	50	20
12-19-3B$_1$	12-19-3B	1.0142	50	20
12-19-3B$_2$	12-19-3B	1.0091	50	20
12-19-3B$_3$	12-19-3B	1.0165	50	20

[a] Contained 2 mL of IS solution.

4.3.3 ~ 64 mg/mL Formulation

The contents of the ~ 64 mg/mL formulation containers (12-19-2T, 12-19-2M, and 12-19-2B) were resuspended using a Polytron homogenizer for ~ 2 minutes (Setting 5) followed by mixing using stir bars and magnetic stir plates. While stirring, triplicate aliquots (~ 1 g; accurately weighed) from each sample were transferred into individual 50-mL volumetric flasks. The contents of each flask were diluted to volume with acetone and mixed by inversion. A 7-mL aliquot from each flask was transferred into individual 25-mL volumetric flasks each containing 2 mL of IS solution. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 6).

Table 6. Sample Preparation of ~ 64 mg/mL Formulation Prepared at ILS, Inc.

Formulation sample	Formulation container	Formulation aliquot (g)	Volume (mL)	Dilution aliquot (mL)	Final volume (mL)[a]	Target concentration (mg/mL)
12-19-2T$_1$	12-19-2T	1.0032	50	7	25	64
12-19-2T$_2$	12-19-2T	1.0149	50	7	25	64
12-19-2T$_3$	12-19-2T	1.0029	50	7	25	64
12-19-2M$_1$	12-19-2M	1.0072	50	7	25	64
12-19-2M$_2$	12-19-2M	1.0111	50	7	25	64
12-19-2M$_3$	12-19-2M	1.0102	50	7	25	64
12-19-2B$_1$	12-19-2B	1.0196	50	7	25	64
12-19-2B$_2$	12-19-2B	1.0046	50	7	25	64
12-19-2B$_3$	12-19-2B	1.0087	50	7	25	64

[a] Contained 2 mL of IS solution.

4.3.4 ~ 200 mg/mL Formulation

The contents of the ~ 200 mg/mL formulation containers (12-19-1T, 12-19-1M, and 12-19-1B) were resuspended using a Polytron homogenizer for ~ 2 minutes (Setting 5) followed by mixing using stir bars and magnetic stir plates. While stirring, triplicate aliquots (~ 1 g; accurately weighed) from each sample were transferred into individual 50-mL volumetric flasks.

The contents of each flask were diluted to volume with acetone and mixed by inversion. A 5-mL aliquot from each flask was transferred into individual 50-mL volumetric flasks each containing 2 mL of IS solution. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 7).

Table 7. Sample Preparation of ~ 200 mg/mL Formulation Prepared at ILS, Inc.

Formulation sample	Formulation container	Formulation aliquot (g)	Volume (mL)	Dilution aliquot (mL)	Final volume (mL)[a]	Target concentration (mg/mL)
12-19-1T$_1$	12-19-1T	1.0087	50	5	50	200
12-19-1T$_2$	12-19-1T	1.0097	50	5	50	200
12-19-1T$_3$	12-19-1T	1.0105	50	5	50	200
12-19-1M$_1$	12-19-1M	1.0120	50	5	50	200
12-19-1M$_2$	12-19-1M	1.0063	50	5	50	200
12-19-1M$_3$	12-19-1M	1.0692	50	5	50	200
12-19-1B$_1$	12-19-1B	1.0250	50	5	50	200
12-19-1B$_2$	12-19-1B	1.0046	50	5	50	200
12-19-1B$_3$	12-19-1B	1.0073	50	5	50	200

[a] Contained 2 mL of IS solution.

4.4 Formulation Density Determination

The density of each formulation was determined by transferring aliquots of formulation into three individual pre-weighed 10-mL volumetric flasks each for the ~ 0, ~ 20, and ~ 64 mg/mL formulations. Once the volumetric flasks were filled to volume, the weights of the filled flasks were recorded. For the ~ 200 mg/mL formulation, three individual pre-weighed 10-mL graduated cylinders were filled to the 10-mL mark and the weights of the cylinders were recorded. The density of each formulation was determined by dividing the individual weights of the filled vessels (g) by 10 mL and the average densities were determined using commonly accepted techniques (see Table 8).

Table 8. Formulation Density Determination

Target formulation concentration (mg/mL)	Formulation sample	Weight of filled vessel (g)	Volume (mL)	Average density (g/mL)
0	N135-11-121-32212	9.1990	10	0.92084
	N135-11-121-32212	9.2101	10	
	N135-11-121-32212	9.2162	10	
20	12-19-3T	9.2467	10	0.92586
	12-19-3M	9.2732	10	
	12-19-3B	9.2559	10	
64	12-19-2T	9.2054	10	0.92843
	12-19-2M	9.3162	10	
	12-19-2B	9.3312	10	
200	12-19-1T	9.6373	10	0.94857
	12-19-1M	9.3835	10	
	12-19-1B	9.4363	10	

4.5 Sample Analysis

4.5.1 Mobile Phase Preparation

For Mobile Phase A, ~ 950 mL of purified water and ~ 50 mL of methanol were added to a container. The solution was mixed by inversion, filtered, and degassed by sonication under vacuum. For Mobile Phase B, ~ 1,900 mL of methanol and ~ 100 mL of purified water were added to a container. The solution was mixed by inversion, filtered, and degassed by sonication under vacuum. NOTE: Mobile phase preparation volumes varied during preparation, but the composition ratio remained constant.

4.5.2 Instrument System and Parameters

Aliquots of the spiked matrix standards, blanks, and formulation samples were filtered through 0.45 µm PTFE syringe filters into individual LC autosampler vials and analyzed using the HPLC/UV system and parameters in Table 9. Spiked matrix standard A_{41} was used for system suitability and check standards.

Table 9. HPLC/UV System and Parameters

Instrument:	Waters 2695 Separations Module
Column:	Waters XTerra RP-18, 250 x 4.6 mm, 5 µm
Column Temperature:	30°C
Detector:	UV, Waters 2487 Dual Absorbance
Wavelength:	272 nm
Detector Range:	0.7 AUFS
Mobile Phase:	Gradient; Mobile Phase A: 95/5 Water/Methanol; Mobile Phase B: 95/5 Methanol/Water
Gradient Program:	Time %A %B 0 20 80 20 0 100 22 20 80 30 20 80
Flow Rate:	1.0 mL/min
Injection Volume:	10 µL
Run Time:	30 min
Data System:	TotalChrom, Version: 6.3.0 and PE NCI902 interface
Retention Time:	Avobenzone Keto Tautomer: ~ 6.9 min n-Decanophenone (IS): ~ 13.1 min Avobenzone Enol Tautomer: ~ 16.2 min

4.6 Calculations

1. The peak area ratio (PAR) was calculated as follows:

$$PAR = \frac{\text{Peak Area (Avobenzone Enol)} + \text{Peak Area (Avobenzone Keto)}}{\text{Peak Area (IS)}}$$

NOTE: for the ~ 64 mg/mL formulation samples, the IS Peak Area was divided by 2 before the PAR was calculated, see Section 4.7 for further explanation.

2. The slope, y-intercept, and correlation coefficient were calculated from a non-weighted linear regression analysis of the spiked matrix standard curve by relating the PAR of each spiked matrix standard with its corresponding expected analytical concentration.

3. Using the slope and y-intercept determined from the spiked matrix standard curve and the PAR for each spiked matrix standard, the determined formulation concentration of each spiked matrix standard was calculated using the following equation:

$$\text{Determined formulation concentration (mg/mL)} = \frac{[\text{PAR} - (\text{y - intercept})]}{\text{slope}} \times \frac{50\ \text{mL}}{1\ \text{g}} \times \frac{0.92278\ \text{g}}{1\ \text{mL}} \times \frac{1\ \text{mg}}{1{,}000\ \mu\text{g}}$$

4. For the spiked matrix standard curve, method accuracy, expressed as percent relative error (% RE), was calculated as follows (acceptable criterion: % RE ≤ 10):

$$\%\ \text{RE} = \frac{(D - E)}{E} \times 100$$

where: D = determined formulation concentration

E = expected formulation concentration

5. Using the slope and y-intercept determined from the spiked matrix standard curve and the PAR for each sample, the determined formulation concentration of each formulation sample was calculated using the following equation:

$$\text{Determined formulation concentration (mg/mL)} = \frac{[\text{PAR} - (\text{y - intercept})]}{\text{slope}} \times \text{Dilution Factor}$$

$$\text{For ~0 mg/mL formulations, Dilution Factor} = \frac{0.92084\ \text{g}}{1\ \text{mL}} \times \frac{50\ \text{mL}}{\text{Sample Weight (g)}} \times \frac{1\ \text{mg}}{1{,}000\ \mu\text{g}}$$

$$\text{For ~20 mg/mL formulations, Dilution Factor} = \frac{0.92586\ \text{g}}{1\ \text{mL}} \times \frac{50\ \text{mL}}{\text{Sample Weight (g)}} \times \frac{1\ \text{mg}}{1{,}000\ \mu\text{g}}$$

$$\text{For ~64 mg/mL formulations, Dilution Factor} = \frac{0.92843\ \text{g}}{1\ \text{mL}} \times \frac{50\ \text{mL}}{\text{Sample Weight (g)}} \times \frac{25\ \text{mL}}{7\ \text{mL}} \times \frac{1\ \text{mg}}{1{,}000\ \mu\text{g}}$$

$$\text{For ~200 mg/mL formulations, Dilution Factor} = \frac{0.94857\ \text{g}}{1\ \text{mL}} \times \frac{50\ \text{mL}}{\text{Sample Weight (g)}} \times \frac{50\ \text{mL}}{5\ \text{mL}} \times \frac{1\ \text{mg}}{1{,}000\ \mu\text{g}}$$

6. The average determined concentration for each formulation was calculated using commonly accepted techniques.

7. The % of Target for the formulations was calculated as follows:

$$\%\ \text{of Target} = \frac{\text{Determined Formulation Concentration (mg/mL)}}{\text{Target Concentration (mg/mL)}} \times 100$$

8. The average % of Target for each formulation was calculated using commonly accepted techniques.

9. The calculated determined formulation concentration (D) was compared to the expected formulation concentration (E) and expressed as a percentage as follows:

$$\%\,D/E = \frac{D}{E} \times 100$$

10. System suitability parameters were calculated for system precision, peak tailing (T), theoretical plates (N), and resolution (R) according to USP guidelines.[6] System precision was calculated using the mean PAR from six replicate injections of a mid-range spiked matrix standard. Peak tailing (at 5% peak height), theoretical plates (tangential method), and resolution were calculated from single injections of a mid-range spiked matrix standard.

11. Sample mean ($\bar{X}$), standard deviation (s), and percent relative standard deviation (% RSD) were calculated using commonly accepted techniques.

4.7 Results

Formulations of avobenzone in corn oil at ~ 0, ~ 20, ~ 64, and ~ 200 mg/mL were received from ILS, Inc., analyzed for formulation concentration at MRI. For sample analysis, a spiked matrix standard curve was prepared to cover a formulation range of 3.8591 to 50.696 mg/mL (analytical range of 83.640 to1,098.8 µg/mL). The curve proved to be linear (r = 0.99937) and accurate (% RE from –2.9 to 6.9). The spiked matrix curve data is presented in Table 21 and the system suitability data is presented in Table 24.

Results from the analysis of ILS, Inc., prepared formulations indicated that the ~ 0 mg/mL formulation (N135-11-121-32212) did not contain any detectable avobenzone (see Table 25). A representative chromatogram is displayed in Figure 1.

The three samples of ~ 20 mg/mL formulation (12-19-3T, 12-19-3M, and 12-19-3B) were all within criteria (< 10% of target) with average percent of target values of 102.7%, 102.6%, and 105.0% for the 12-19-3T, 12-19-3M, and 12-19-3B samples, respectively. The ~ 20 mg/mL formulation was considered to be homogeneous since the RSD of the samples was ≤ 3.6%. The homogeneity and formulation data are presented in Table 25 and a representative chromatogram is displayed in Figure 1.

The three samples of the ~ 64 mg/mL formulation (12-19-2T, 12-19-2M, and 12-19-2B), were all within criteria with average percent of target values of 98.5%, 97.3%, and 97.2% for the 12-19-2T, 12-19-2M, and 12-19-2B samples, respectively. The ~ 64 mg/mL formulation was considered homogeneous since the RSD of the samples was ≤ 0.9%. During analysis, the formulation appeared to be around ~ 32 mg/mL. To further evaluate, the ~ 64 mg/mL formulation was re-analyzed using a different dilution scheme during sample preparation (see Section 5). After re-analysis, it was determined that the amount of IS was doubled in each sample, which was corrected by dividing the IS peak area by two before the PAR was calculated.

[6] *United States Pharmacopeia* [621] Chromatography, (2007), official from May 1, 2007, 30th Edition, pp. 243-256.

The homogeneity and formulation data, with the correction factor applied, are presented in Table 25 and a representative chromatogram is displayed in Figure 1.

The three samples of the ~ 200 mg/mL formulation (12-19-1T, 12-19-1M, and 12-19-1B) were all within criteria with average percent of target values of 104.5%, 105.4%, and 104.4% for the 12-19-1T, 12-19-1M, and 12-19-1B samples, respectively. The ~ 200 mg/mL formulation was considered homogeneous since the RSD of the samples was ≤ 1.1%. The homogeneity and formulation data are presented in Table 25 and a representative chromatogram is displayed in Figure 1.

5. Re-Analysis of the ILS, Inc., Prepared Formulation at ~ 64 mg/mL

To determine the cause of the apparent low determined concentration of the ~ 64 mg/mL formulation (see Section 4.7), formulation re-analysis was performed on the ~ 64 mg/mL formulation samples (12-19-2T, 12-19-2M, and 12-19-2B) using a different dilution scheme during sample preparation to confirm the accuracy of the sample preparation method.

5.1 Standards Preparation

5.1.1 IS Solution

The IS solution was prepared as described in Section 4.1.1.

5.1.2 Stock Solutions

Two stock solutions (Stocks A and B) were prepared by accurately weighing and transferring 550.45 mg (Stock A) and 416.52 mg (Stock B) into individual 50-mL volumetric flasks. The contents of each flask were diluted to volume with acetone and mixed by inversion for expected concentrations of 11.009 mg/mL (Stock A) and 8.3304 mg/mL (Stock B).

5.1.3 Intermediate Solutions

Intermediate solutions (IB_1 to IA_6) were prepared by transferring aliquots from alternating stock solutions (Stocks A and B) into individual volumetric flasks. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 10).

NOTE: Stock B was used as IB_5 and Stock A was used as IA_6.

Table 10. Intermediate Solutions Preparation for Formulation Re-analysis at ~ 64 mg/mL

Intermediate solution	Stock solution	Stock solution aliquot (mL)	Volume (mL)	Expected analytical concentration (µg/mL)
IB_1	B	5	50	833.04
IA_2	A	10	50	2,201.8
IB_3	B	10	25	3,332.2
IA_4	A	10	25	4,403.6
IB_5	B	NA	NA	8,330.4
IA_6	A	NA	NA	11,009

5.1.4 Spiked Matrix Standards

Spiked matrix standards (B_{11} to A_{61}) were prepared by transferring 5 mL aliquots of the intermediate solutions (Section 5.1.3) into individual 50-mL volumetric flasks each containing an ~ 1 g aliquot of corn oil and 2 mL of IS solution. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 11).

Table 11. Spiked Matrix Standards Preparation for Formulation Re-analysis at ~ 64 mg/mL

Spiked matrix standard	Intermediate solution	Intermediate solution aliquot (mL)	Matrix added (~ g)	Final volume (mL)[a]	Expected analytical concentration (µg/mL)	Expected formulation concentration (mg/mL)[b]
B_{11}	IB_1	5	1	50	83.304	3.8436
A_{21}	IA_2	5	1	50	220.18	10.159
B_{31}	IB_3	5	1	50	333.22	15.374
A_{41}	IA_4	5	1	50	440.36	20.318
B_{51}	IB_5	5	1	50	833.04	38.436
A_{61}	IA_6	5	1	50	1,100.9	50.794

[a] Contained 2 mL of IS solution.
[b] Density of matrix = 0.92278 g/mL (determined in the associated DFD, CHEM10987[3]).

5.2 Blanks Preparation

5.2.1 Reagent Blank

The reagent blank was prepared as described in Section 4.2.1.

5.2.2 IS Blank

The IS blank was prepared as described in Section 4.2.2.

5.2.3 Matrix Blank

A matrix blank (C_{01}) was prepared as described in Section 4.2.3. A matrix blank with IS (C_{02}) was not prepared in this re-analysis.

5.3 Formulation Sample Preparation

The contents of the ~ 64 mg/mL formulation containers (12-19-2T, 12-19-2M, and 12-19-2B) were resuspended using a Polytron homogenizer for ~ 2 minutes (Setting 5) followed by mixing using stir bars and magnetic stir plates. While stirring, triplicate aliquots (~ 1 g; accurately weighed) from each sample were transferred into individual 50-mL volumetric flasks. The contents of each flask were diluted to volume with acetone and mixed by inversion. A 10-mL aliquot from each flask was transferred into individual 25-mL volumetric flasks each containing 2 mL of IS solution. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 12).

Table 12. Sample Preparation for the Re-analysis of the ~ 64 mg/mL Formulation Prepared at ILS, Inc.

Formulation sample	Formulation container	Formulation aliquot (g)	Volume (mL)	Dilution aliquot (mL)	Final volume (mL)[a]	Target concentration (mg/mL)
R-12-19-2T$_1$	12-19-2T	1.0169	50	10	25	64
R-12-19-2T$_2$	12-19-2T	1.0167	50	10	25	64
R-12-19-2T$_3$	12-19-2T	1.0348	50	10	25	64
R-12-19-2M$_1$	12-19-2M	1.0042	50	10	25	64
R-12-19-2M$_2$	12-19-2M	1.0048	50	10	25	64
R-12-19-2M$_3$	12-19-2M	1.0065	50	10	25	64
R-12-19-2B$_1$	12-19-2B	1.0003	50	10	25	64
R-12-19-2B$_2$	12-19-2B	1.0146	50	10	25	64
R-12-19-2B$_3$	12-19-2B	1.0258	50	10	25	64

[a] Contained 2 mL of IS solution.

5.4 Sample Analysis

5.4.1 Mobile Phase Preparation

The mobile phase for analysis was prepared as described in Section 4.5.1.

5.4.2 Instrument System and Parameters

Aliquots of the spiked matrix standards, blanks, and formulation samples were filtered through 0.45 µm PTFE syringe filters into individual LC autosampler vials and analyzed using the HPLC/UV system and parameters in Table 9 (Section 4.5.2). Spiked matrix standard A_{41} was used for system suitability and check standards.

5.5 Calculations

See Section 4.6 for calculations with the following exception:

The dilution factor used for the determined formulation concentration of the samples (Equation 5) was:

$$\text{For} \sim 64\ \text{mg/mL formulations, Dilution Factor} = \frac{0.92843\ \text{g}}{1\ \text{mL}} \times \frac{50\ \text{mL}}{\text{Sample Weight (g)}} \times \frac{25\ \text{mL}}{10\ \text{mL}} \times \frac{1\ \text{mg}}{1{,}000\ \mu\text{g}}$$

5.6 Results

The ~ 64 mg/mL formulation of avobenzone in corn oil received from ILS, Inc., was re-analyzed for avobenzone concentration using a different dilution scheme during sample preparation to confirm the sample preparation method. For analysis, a spiked matrix standard curve was prepared to cover a formulation range of 3.8436 to 50.794 mg/mL (analytical range of 83.304 to 1,100.9 µg/mL). The curve proved to be linear (r = 0.999813) and accurate (% RE from –1.4 to 7.9). The spiked matrix curve data is presented in Table 22 and the system suitability data is presented in Table 24.

Results from the ~ 64 mg/mL formulation re-analysis indicated that the samples were all within criteria (< 10% of target concentration) with average percent of target values of 99.9%, 98.3%, and 98.6% for the 12-19-2T, 12-19-2M, and 12-19-2B samples, respectively. The ~ 64 mg/mL formulation was considered homogeneous since the RSD of the samples was ≤ 1.0%. The formulation data is presented in Table 26.

6. Analysis of MRI Prepared Formulation at ~ 64 mg/mL

To confirm the formulation preparation method used for the ~ 64 mg/mL formulation, MRI prepared a formulation of avobenzone in corn oil at ~ 64 mg/mL and analyzed the formulation for formulation concentration and homogeneity.

6.1 Formulation Preparation

An ~ 64 mg/mL formulation of avobenzone in corn oil was prepared by accurately weighing and transferring 64.0089 g of avobenzone to a 2-L beaker. Assuming the density of corn oil is 0.92278 g/mL (as determined in the associated DFD assignment, CHEM10987[3]), 922.8 g of corn oil was added to the beaker to make an ~ 1 L formulation. The formulation was mixed using a combination of stir bar and stir plate, Polytron homogenization, and sonication (see Table 13).

Table 13. Mixing Scheme for Formulation Preparation at ~ 64 mg/mL

Mixing method	Length of mixing (min)	Formulation appearance
Stirring	20	Large white particles present throughout
Polytron Homogenization	2	Large particles still visible
Stirring	10	No change
Sonication	5	No change
Stirring	5	No change
Polytron Homogenization while Stirring	3	Fewer clumps of particles observed
Stirring	5	No change
Sonication	5	No change
Stirring	5	No change
Polytron Homogenization while Stirring	3	No change
Stirring	15	No change
Polytron Homogenization while Stirring	2	No change
Stirring	15	No change
Sonication	5	No change
Stirring	5	Formulation looked like a uniform suspension
Polytron Homogenization	2	Clumps started to form again
Stirring	4	Clumps visible
Sonication	10	Clumps less visible
Stirring	5	Formulation looked like a uniform suspension

6.2 Formulation Sample Preparation

After the formulation was determined to be a uniform suspension, triplicate aliquots (~ 1 g; accurately weighed) from each of the top, middle and bottom of the formulation container were transferred into individual 50-mL volumetric flasks while the suspension was being stirred with a stir bar and magnetic stir plate. The contents of each flask were diluted to volume with acetone and mixed by inversion. A 10-mL aliquot from each flask was transferred into individual 25-mL volumetric flasks each containing 2 mL of IS solution. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 14).

Table 14. Sample Preparation of the MRI Prepared ~ 64 mg/mL Formulation

Formulation sample	Formulation aliquot (g)	Volume (mL)	Dilution aliquot (mL)	Final volume (mL)[a]	Expected analytical concentration (µg/mL)	Expected formulation concentration (mg/mL)
64-T_1	1.0156	50	10	25	554.428	64.0075
64-T_2	1.0072	50	10	25	549.843	64.0075
64-T_3	1.0105	50	10	25	551.644	64.0075
64-M_1	1.0246	50	10	25	559.342	64.0075
64-M_2	1.0086	50	10	25	550.607	64.0075
64-M_3	1.0315	50	10	25	563.108	64.0075
64-B_1	1.0028	50	10	25	547.441	64.0075
64-B_2	1.0159	50	10	25	554.592	64.0075
64-B_3	1.0092	50	10	25	550.935	64.0075

[a] Contained 2 mL of IS solution.

6.3 Formulation Density Determination

The density of the ~ 64 mg/mL formulation was determined by transferring aliquots of formulation into three individual pre-weighed 10-mL graduated cylinders. The cylinders were filled to the 10-mL mark and the weights of the cylinders were recorded. The density was determined by dividing the individual weights of the filled cylinders (g) by 10 mL and the average densities were determined using commonly accepted techniques (see Table 15).

Table 15. Formulation Density Determination—MRI Prepared Formulation at ~ 64 mg/mL

Target formulation concentration (mg/mL)	Weight of filled cylinder (g)	Volume (mL)	Average density (g/mL)
64	9.2941	10	0.93799
	9.4766	10	
	9.3691	10	

6.4 Standards Preparation

6.4.1 IS Solution

IS solution was prepared as described in Section 4.1.1.

6.4.2 Stock Solutions

Two stock solutions (Stocks A and B) were prepared by accurately weighing and transferring 550.93 mg (Stock A) and 416.51 mg (Stock B) into individual 50-mL volumetric flasks. The contents of each flask were diluted to volume with acetone and mixed by inversion for expected concentrations of 11.019 mg/mL (Stock A) and 8.3302 mg/mL (Stock B).

6.4.3 Intermediate Solutions

Intermediate solutions (IB_1 to IA_6) were prepared by transferring aliquots from alternating stock solutions (Stocks A and B) into individual volumetric flasks. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 16).

NOTE: Stock B was used as IB_5 and Stock A was used as IA_6.

Table 16. Intermediate Solutions Preparation—MRI Prepared Formulation Analysis

Intermediate solution	Stock solution	Stock solution aliquot (mL)	Volume (mL)	Expected analytical concentration (µg/mL)
IB_1	B	5	50	833.02
IA_2	A	10	50	2,203.7
IB_3	B	10	25	3,332.1
IA_4	A	10	25	4,407.4
IB_5	B	NA	NA	8,330.2
IA_6	A	NA	NA	11,019

6.4.4 Spiked Matrix Standards

Spiked matrix standards (B_{11} to A_{61}) were prepared by transferring 5-mL aliquots of the intermediate solutions (see Section 6.4.3) into individual 50-mL volumetric flasks each containing an ~ 1 g portion of corn oil and 2 mL of IS solution. The contents of each flask were diluted to volume with acetone and mixed by inversion (see Table 17).

Table 17. Spiked Matrix Standards Preparation for ILS, Inc., Prepared Formulation Analysis

Spiked matrix standard	Intermediate solution	Intermediate solution aliquot (mL)	Matrix added (~ g)	Final volume (mL)[a]	Expected analytical concentration (µg/mL)	Expected formulation concentration (mg/mL)[b]
B_{11}	IB_1	5	1	50	83.302	3.8435
A_{21}	IA_2	5	1	50	220.37	10.168
B_{31}	IB_3	5	1	50	333.21	15.374
A_{41}	IA_4	5	1	50	440.74	20.335
B_{51}	IB_5	5	1	50	833.02	38.435
A_{61}	IA_6	5	1	50	1,101.9	50.839

[a] Contained 2 mL of IS solution.
[b] Density of matrix = 0.92278 g/mL (determined in the associated DFD, CHEM10987[3]).

6.5 Blanks Preparation

6.5.1 Reagent Blank

The reagent blank was prepared as described in Section 4.2.1.

6.5.2 IS Blank

The IS blank was prepared as described in Section 4.2.2.

6.5.3 Matrix Blank

Matrix blanks (C_{01} and C_{02}) was prepared as described in Section 4.2.3.

6.6 Sample Analysis

6.6.1 Mobile Phase Preparation

Mobile phase was prepared as described in Section 4.5.1.

6.6.2 Instrument System and Parameters

Aliquots of the spiked matrix standards, blanks, and formulations samples were filtered through 0.45 µm PTFE syringe filters into individual LC autosampler vials and analyzed using the HPLC/UV system and parameters in Table 9 (Section 4.5.2). Spiked matrix standard A_{41} was used for system suitability and check standards.

6.7 Calculations

See Section 4.6 for calculations with the following exception:

The dilution factor used for the determined formulation concentration of the samples (Equation 5) was:

$$\text{For} \sim 64\ \text{mg/mL formulations, Dilution Factor} = \frac{0.93799\ \text{g}}{1\ \text{mL}} \times \frac{50\ \text{mL}}{\text{Sample Weight (g)}} \times \frac{25\ \text{mL}}{10\ \text{mL}} \times \frac{1\ \text{mg}}{1{,}000\ \mu\text{g}}$$

6.8 Results

A formulation of avobenzone in corn oil at ~ 64 mg/mL was prepared and analyzed at MRI for formulation concentration and homogeneity. For analysis, a spiked matrix standard curve was prepared to cover a formulation range of 3.8435 to 50.839 mg/mL (analytical range of 83.302 to 1,1101.9 µg/mL). The curve proved to be linear (r = 0.99982) and accurate (% RE from –3.9 to 4.6). However, the tailing factor of the enol avobenzone tautomer was found to be slightly out of range at 2.305 (upper limit criterion = 2.0). Even though the enol tautomer tailing factor was slightly out of range, all other criteria were met so it did not affect the results of this study. The spiked matrix standard curve data is presented in Table 23 and the system suitability data is presented in Table 24.

The results of formulation preparation analysis indicated that the ~ 64 mg/mL formulation was within criteria with average percent of target values of 93.6%, 92.8%, and 91.4% for the top, middle, and bottom of the formulation, respectively. The formulation was considered to be

homogeneous since the RSD of samples taken from the top, middle, and bottom of the formulations was ≤ 2.5%. The homogeneity and formulation data are presented in Table 27.

7. Conclusions

In this study, formulations of avobenzone in corn oil at ~ 0, ~ 20, ~ 64, and ~ 200 mg/mL were prepared by ILS, Inc., and analyzed for formulation concentration at MRI using a validated HPLC/UV method. In addition, a ~ 64 mg/mL formulation was prepared and analyzed for avobenzone concentration and formulation homogeneity at MRI.

For the ILS, Inc. prepared formulations, all formulation average values were within ±5.4% of the target concentration as shown in Table 18.

Table 18. Summary of Analysis Results of ILS, Inc., Prepared Formulations

ILS, Inc. sample name	MRI sample name	Target formulation concentration (mg/mL)	Average % of target
N135-11-121-32212	N135-11-121-32212$_1$ N135-11-121-32212$_2$ N135-11-121-32212$_3$	0	NA
12-19-1T	12-19-1T$_1$, 12-19-1T$_2$, 12-19-1T$_3$	200	104.5 ± 0.5 (s)
12-19-1M	12-19-1M$_1$, 12-19-1M$_2$, 12-19-1M$_3$		105.4 ± 0.8 (s)
12-19-1B	12-19-1B$_1$, 12-19-1B$_2$, 12-19-1B$_3$		104.4 ± 1.2 (s)
12-19-2T	12-19-2T$_1$, 12-19-2T$_2$, 12-19-2T$_3$	64	98.5 ± 0.9 (s)
12-19-2M	12-19-2M$_1$, 12-19-2M$_2$, 12-19-2M$_3$		97.3 ± 0.4 (s)
12-19-2B	12-19-2B$_1$, 12-19-2B$_2$, 12-19-2B$_3$		97.2 ± 0.3 (s)
12-19-3T	12-19-3T$_1$, 12-19-3T$_2$, 12-19-3T$_3$	20	102.7 ± 0.8 (s)
12-19-3M	12-19-3M$_1$, 12-19-3M$_2$, 12-19-3M$_3$		102.6 ± 1.1 (s)
12-19-3B	12-19-3B$_1$, 12-19-3B$_2$, 12-19-3B$_3$		105.0 ± 3.8 (s)

During analysis of the ~ 64 mg/mL formulation, the preliminary results showed the formulation was ~ 32 mg/mL. To further evaluate, the formulation was re-analyzed using a different dilution scheme to confirm the sample preparation method. After re-analysis, it was determined that the amount of IS was doubled in each sample and a correction was made to the calculations. The corrected data is presented in Tables 18 and 19. When the correction factor was applied, the formulation percent of target was ±1.7% for the three samples of formulation at ~ 64 mg/mL.

Table 19. Summary of Re-analysis Results of ILS, Inc., Prepared ~ 64 mg/mL Formulation

ILS, Inc. sample name	MRI sample name	Target formulation concentration (mg/mL)	Average % of target
12-19-2T	R-12-19-2T$_1$, R-12-19-2T$_2$, R-12-19-2T$_3$	64	99.9 ± 0.7 (s)
12-19-2M	R-12-19-2M$_1$, R-12-19-2M$_2$, R-12-19-2M$_3$		98.3 ± 0.6 (s)
12-19-2B	R-12-19-2B$_1$, R-12-19-2B$_2$, R-12-19-2B$_3$		98.6 ± 0.8 (s)

To confirm the ~64 mg/mL formulation preparation method, MRI prepared and analyzed a formulation at ~64 mg/mL. The formulation percent of target was ±8.6% for samples taken from the top, middle, and bottom of the formulation container.

Table 20. Summary of Results of MRI Prepared Formulation at ~64 mg/mL

MRI sample name	Formulation sampling location	Target formulation concentration (mg/mL)	Average % of target
64-T_1, 64-T_2, 64-T_3	Top	64	93.6 ± 0.4 (s)
64-M_1, 64-M_2, 64-M_3	Middle	64	92.8 ± 0.9 (s)
64-B_1, 64-B_2, 64-B_3	Bottom	64	91.4 ± 2.3 (s)

8. Contributors

The personnel contributing to this study were [redacted]

Table 21. Spiked Matrix Curve of Avobenzone in Corn Oil—Analysis of Formulations Received From ILS, Inc.

Linear Regression Parameters							
				Spiked Matrix Standard Data			
Correlation coefficient				0.99937			
Slope				0.012899			
Y-intercept				–0.17384			
Spiked matrix standard	Expected formulation concentration (E) (mg/mL)	Expected analytical concentration (µg/mL)	PAR[a]	Determined analytical concentration (µg/mL)[b]	Determined formulation concentration (D) (mg/mL)[c]	% RE[d]	% D/E[e]
B_{11}	3.8591	83.640	0.978957	89.371	4.1235	6.9	106.9
A_{21}	10.139	219.75	2.661044	219.78	10.140	0.0	100.0
B_{31}	15.436	334.56	4.098711	331.23	15.283	–1.0	99.0
A_{41}	20.278	439.50	5.555509	444.17	20.494	1.1	101.1
B_{51}	38.591	836.40	10.301894	812.14	37.471	–2.9	97.1
A_{61}	50.696	1,098.8	14.221332	1,116.0	51.491	1.6	101.6

[a] PAR = Peak Area Ratio = (Avobenzone Enol Peak Area + Avobenzone Keto Peak Area)/ IS Peak Area.
[b] Determined analytical concentration (µg/mL) = (PAR – y-intercept)/slope.
[c] Determined formulation concentration (mg/mL) = (PAR – y-intercept)/slope * dilution factor.
[d] % RE = (D - E)/ E * 100.
[e] % D/E = D / E * 100.

Table 22. Spiked Matrix Curve of Avobenzone in Corn Oil—Re-analysis of ILS, Inc., Received Formulation at ~ 64 mg/mL

Linear Regression Parameters							
				Spiked Matrix Standard Data			
Correlation coefficient				0.999813			
Slope				0.013411			
Y-intercept				–0.267154			
Spiked matrix standard	Expected formulation concentration (E) (mg/mL)	Expected analytical concentration (µg/mL)	PAR[a]	Determined analytical concentration (µg/mL)[b]	Determined formulation concentration (D) (mg/mL)[c]	% RE[d]	% D/E[e]
B_{11}	3.8436	83.304	0.937880	89.854	4.1458	7.9	107.9
A_{21}	10.159	220.18	2.649894	217.51	10.036	–1.2	98.8
B_{31}	15.374	333.22	4.195519	332.76	15.353	–0.1	99.9
A_{41}	20.318	440.36	5.622562	439.17	20.263	–0.3	99.7
B_{51}	38.436	833.04	10.746821	821.26	37.892	–1.4	98.6
A_{61}	50.794	1,100.9	14.625894	1,110.5	51.238	0.9	100.9

[a] PAR = Peak Area Ratio = (Avobenzone Enol Peak Area + Avobenzone Keto Peak Area)/ IS Peak Area.
[b] Determined analytical concentration (µg/mL) = (PAR – y-intercept)/slope.
[c] Determined formulation concentration (mg/mL) = (PAR – y-intercept)/slope * dilution factor.
[d] % RE = (D - E) / E * 100.
[e] % D/E = D / E * 100.

Table 23. Spiked Matrix Curve of Avobenzone in Corn Oil—MRI Formulation Preparation and Analysis at ~ 64 mg/mL

Linear Regression Parameters							
				Spiked Matrix Standard Data			
Correlation coefficient				0.99982			
Slope				0.012188			
Y-intercept				–0.25910			
Spiked matrix standard	Expected formulation concentration (E) (mg/mL)	Expected analytical concentration (µg/mL)	PAR[a]	Determined analytical concentration (µg/mL)[b]	Determined formulation concentration (D) (mg/mL)[c]	% RE[d]	% D/E[e]
B_{11}	3.8435	83.302	0.802485	87.101	4.0187	4.6	104.6
A_{21}	10.168	220.37	2.321199	211.71	9.7680	–3.9	96.1
B_{31}	15.374	333.21	3.854704	337.53	15.573	1.3	101.3
A_{41}	20.335	440.74	5.172797	445.68	20.563	1.1	101.1
B_{51}	38.435	833.02	9.767062	822.63	37.955	–1.2	98.8
A_{61}	50.839	1,101.9	13.244787	1,108.0	51.120	0.6	100.6

[a] PAR = Peak Area Ratio = (Avobenzone Enol Peak Area + Avobenzone Keto Peak Area)/ IS Peak Area.
[b] Determined analytical concentration (µg/mL) = (PAR – y-intercept)/slope.
[c] Determined formulation concentration (mg/mL) = (PAR – y-intercept)/slope * dilution factor.
[d] % RE = (D - E)/ E * 100.
[e] % D/E = D / E * 100.

Table 24. System Suitability Results

Analysis	System precision	Theoretical plates	Tailing factor	Resolution
Method Criteria	RSD ≤ 5.0%	Enol ≥ 9,000 Keto ≥ 5,000 IS ≥ 13,000	Enol = 0.9 ≤ T ≤ 2.0 Keto = 0.9 ≤ T ≤ 1.4 IS = 0.9 ≤ T ≤ 1.7	≥ 4[a] ≥ 13[b]
Analytical Results				
Formulation Analysis ILS, Inc., Prepared Formulations	5.464483 ± 0.040476(s) RSD = 0.7% (n = 6)	Enol = 27,710 Keto = 7,509 IS = 25,819	Enol = 1.507 Keto = 1.134 IS = 1.268	8.760 18.813
Formulation Re-Analysis ILS, Inc., Prepared Formulation at ~ 64 mg/mL	5.544808 ± 0.076386(s) RSD = 1.4% (n = 6)	Enol = 16,998 Keto = 6,685 IS = 16,851	Enol = 1.780 Keto = 1.229 IS = 1.476	6.949 16.509
Analysis of MRI Prepared Formulation at ~ 64 mg/mL	5.159747 ± 0.056822(s) RSD = 1.1% (n = 6)	Enol = 14,484 Keto = 6,688 IS = 15,367	Enol = 2.305[c] Keto = 1.295 IS = 1.684	6.378 16.012

Enol = Avobenzone enol tautomer.
Keto = Avobenzone keto tautomer.
IS = n-decanophenone.
[a] Between avobenzone keto and enol peaks.
[b] Between avobenzone keto and IS peaks.
[c] Tailing factor slightly out of range. Since all other criteria were met, it was concluded that this did not affect the result of the study.

Table 25. Analysis Results of Formulations Prepared at ILS, Inc.

Sample	Target concentration (mg/mL)	Determined formulation concentration (mg/mL)	% of target
N135-11-121-32212$_1$,	0	NA	NA
N135-11-121-32212$_2$	0	NA	NA
N135-11-121-32212$_3$	0	NA	NA
12-19-3T$_1$	20	20.585	102.9
12-19-3T$_2$	20	20.661	103.3
12-19-3T$_3$	20	20.354	101.8
		$\bar{x}$ = 20.533 ± 0.160(s),0.8% RSD	$\bar{x}$ = 102.7 ± 0.8(s), 0.8% RSD
12-19-3M$_1$	20	20.349	101.7
12-19-3M$_2$	20	20.755	103.8
12-19-3M$_3$	20	20.480	102.4
		$\bar{x}$ = 20.528 ± 0.207(s), 1.0% RSD	$\bar{x}$ = 102.6 ± 1.1(s), 1.1% RSD
12-19-3B$_1$	20	20.582	102.9
12-19-3B$_2$	20	20.552	102.8
12-19-3B$_3$	20	21.874	109.4
		$\bar{x}$ = 21.003 ± 0.755(s); 3.6% RSD	$\bar{x}$ = 105.0 ± 3.8(s), 3.6% RSD
Determined Formulation Concentration (mg/mL) = 20.688 ± 0.464 (s); 2.2% RSD (n = 9)			
12-19-2T$_1$	64	62.576	97.8
12-19-2T$_2$	64	63.702	99.5
12-19-2T$_3$	64	62.882	98.3
		$\bar{x}$ = 63.053 ± 0.582(s); 0.9% RSD	$\bar{x}$ = 98.5 ± 0.9(s); 0.9% RSD
12-19-2M$_1$	64	62.069	97.0
12-19-2M$_2$	64	62.165	97.1
12-19-2M$_3$	64	62.585	97.8
		$\bar{x}$ = 62.273 ± 0.274(s); 0.4% RSD	$\bar{x}$ = 97.3 ± 0.4(s); 0.4% RSD
12-19-2B$_1$	64	62.030	96.9
12-19-2B$_2$	64	62.405	97.5
12-19-2B$_3$	64	62.301	97.3
		$\bar{x}$ = 62.245 ± 0.194(s); 0.3% RSD	$\bar{x}$ = 97.2 ± 0.3(s); 0.3% RSD
Determined Formulation Concentration (mg/mL) = 62.524 ± 0.520 (s); 0.8% RSD (n = 9)			
12-19-1T$_1$	200	208.44	104.2
12-19-1T$_2$	200	210.22	105.1
12-19-1T$_3$	200	208.68	104.3
		$\bar{x}$ = 209.11 ± 0.97(s); 0.5% RSD	$\bar{x}$ = 104.5 ± 0.5(s); 0.5% RSD
12-19-1M$_1$	200	211.10	105.6
12-19-1M$_2$	200	211.94	106.0
12-19-1M$_3$	200	209.07	104.5
		$\bar{x}$ = 210.70 ± 1.48(s); 0.7% RSD	$\bar{x}$ = 105.4 ± 0.8(s); 0.8% RSD
12-19-1B$_1$	200	211.34	105.7
12-19-1B$_2$	200	207.36	103.7
12-19-1B$_3$	200	207.30	103.7
		$\bar{x}$ = 208.67 ± 2.32(s); 1.1% RSD	$\bar{x}$ = 104.4 ± 1.2(s); 1.1% RSD
Determined Formulation Concentration (mg/mL) = 209.49 ± 1.73 (s); 0.8% RSD (n = 9)			

NA = not applicable.

Table 26. Re-analysis Results of ~ 64 mg/mL Formulation Prepared at ILS, Inc.

Sample	Target concentration (mg/mL)	Determined formulation concentration (mg/mL)	% of target
R-12-19-2T$_1$	64	63.442	99.1
R-12-19-2T$_2$	64	64.300	100.5
R-12-19-2T$_3$	64	64.129	100.2
		$\bar{x}$ = 63.957 ± 0.454(s)	$\bar{x}$ = 99.9 ± 0.7(s)
		RSD = 0.7%	RSD = 0.7%
R-12-19-2M$_1$	64	62.794	98.1
R-12-19-2M$_2$	64	63.267	98.9
R-12-19-2M$_3$	64	62.620	97.8
		$\bar{x}$ = 62.894 ± 0.335(s)	$\bar{x}$ = 98.3 ± 0.6(s)
		RSD = 0.5%	RSD = 0.6%
R-12-19-2B$_1$	64	63.555	99.3
R-12-19-2B$_2$	64	63.224	98.8
R-12-19-2B$_3$	64	62.524	97.7
		$\bar{x}$ = 63.101 ± 0.526(s)	$\bar{x}$ = 98.6 ± 0.8(s)
		RSD = 0.8%	RSD = 0.8%
Determined Formulation Concentration (mg/mL) = 63.317 ± 0.622 (s); 1.0% RSD (n = 9)			

Table 27. Analysis Results of MRI Formulation Preparation at ~ 64 mg/mL

Sample	Target concentration (mg/mL)	Expected formulation concentration (mg/mL)	Determined formulation concentration (mg/mL)	% of target
64-T$_1$	64	64.0075	59.963	93.7
64-T$_2$	64	64.0075	59.636	93.2
64-T$_3$	64	64.0075	60.194	94.0
			$\bar{x}$ = 59.931 ± 0.280(s)	$\bar{x}$ = 93.6 ± 0.4(s)
			RSD = 0.5%	RSD = 0.4%
64-M$_1$	64	64.0075	59.617	93.1
64-M$_2$	64	64.0075	58.755	91.8
64-M$_3$	64	64.0075	59.931	93.6
			$\bar{x}$ = 59.434 ± 0.609(s)	$\bar{x}$ = 92.8 ± 0.9(s)
			RSD = 1.0%	RSD = 1.0%
64-B$_1$	64	64.0075	59.478	92.9
64-B$_2$	64	64.0075	59.261	92.6
64-B$_3$	64	64.0075	56.865	88.8
			$\bar{x}$ = 58.535 ± 1.450(s)	$\bar{x}$ = 91.4 ± 2.3(s)
			RSD = 2.5%	RSD = 2.5%
Determined Formulation Concentration (mg/mL) = 59.300 ± 1.007 (s); 1.7% RSD (n = 9)				

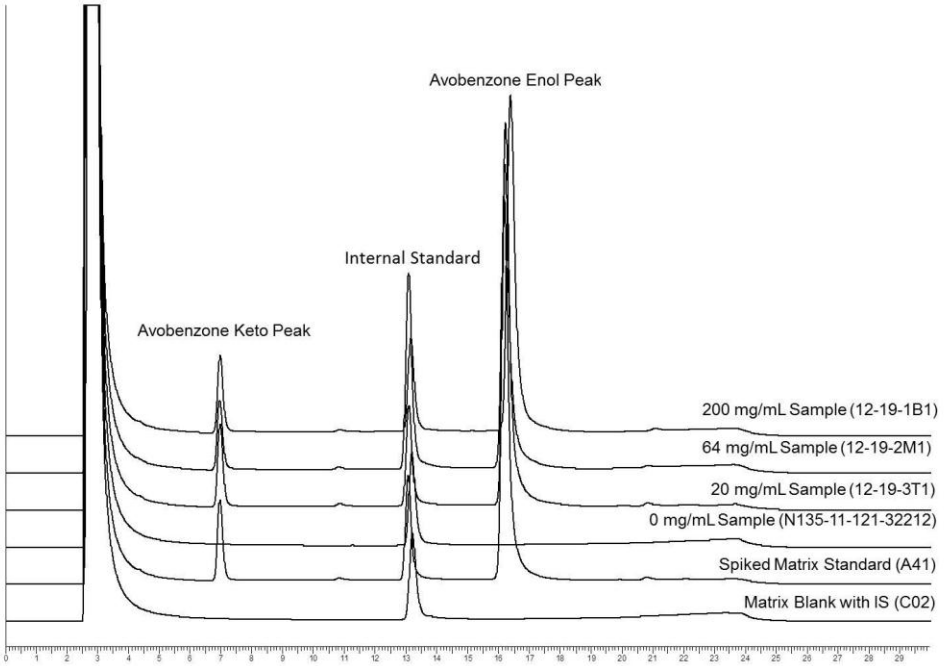


Figure 1. Representative HPLC/UV Chromatograms of Avobenzone in Corn Oil: Matrix Blank With IS, Spiked Matrix Standard, ILS, Inc., Prepared Formulation Samples at ~ 0, ~ 20, ~ 64, and ~ 200 mg/mL

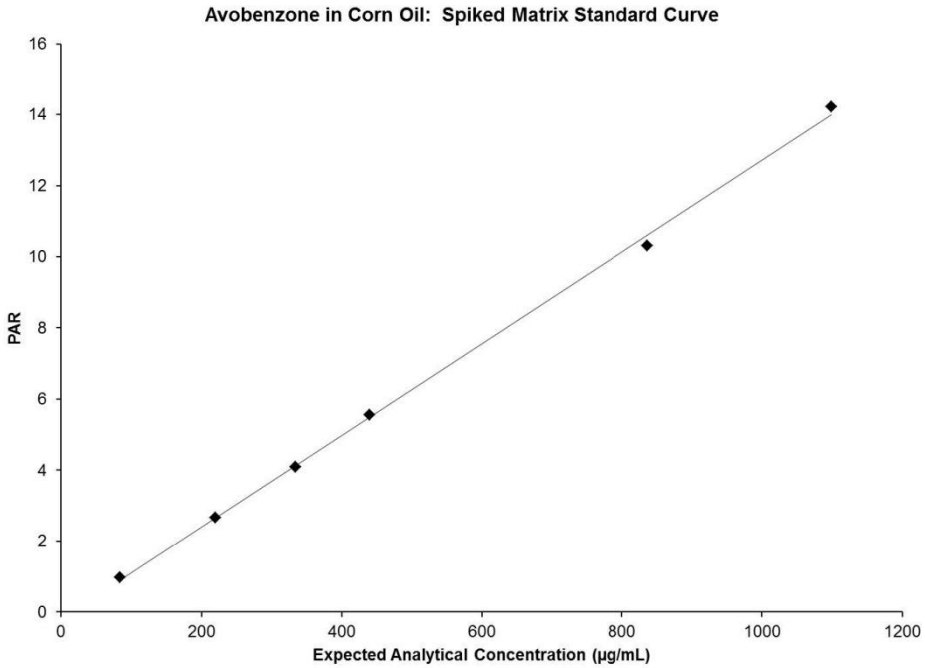


Figure 2. Avobenzone in Corn Oil Spiked Matrix Standard Curve: Analysis of ILS, Inc., Prepared Formulations

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RTI Project 0212839.200.003.076
ChemTask No. CHEM11719
CAS No. 21245-02-3

This pdf is an exact duplicate of the original approved report.
[redacted]
Program Information Coordinator

PADIMATE O

IN CORN OIL

FORMULATION ANALYSIS

Mix Date: March 22, 2012

July 17, 2012

Prepared by:
[redacted]
Task Leader

07-17-12
Date

Approved by:
[redacted]
Reshan Fernando, Ph.D.
Principal Investigator

07-17-12
Date

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P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

PADIMATE O

CAS No.: 21245-02-3

ChemTask No.: CHEM11719

RTI Chemical ID Code: L98

Program Supported: TOX

Analysis Dates: Mar 26-29, 2012

Interim Results Date: Mar 29, 2012

Mix Date: Mar 22, 2012

Lot No. (Vendor): MKBF0590V (Aldrich)

Vendor Purity: 98.3% (Aldrich COA)

Vehicle: Corn oil

Vehicle Lot No.: (Vendor): unknown

Samples Received: 9 x 30 mL and 1 x 100 mL

Dose Formulation Concentrations (RTI Log Nos.):
200 mg/mL (032312-C-01 to -03);
64 mg/mL (032312-C-04 to -06);
20 mg/mL (032312-C-07 to -09);
0 mg/mL (032312-C-10)

Sample Receipt Date: Mar 23, 2012

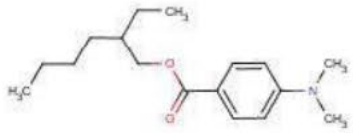
Submitter: ILS

Study Lab (Investigator): ILS ([redacted])

Sample Containers: Amber glass bottles

Receipt Condition: No damage noted

Storage Condition: Room temperature

STRUCTURE	MOL. WT.	MOL. FORMULA
	277.40	$C_{17}H_{27}NO_2$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, a formulation analysis was performed to determine the padimate O concentration and confirm homogeneity of dose formulations prepared in corn oil, submitted by the study lab. In addition a single control sample was received for analysis.

Analyses conducted using a GC/FID method yielded results ranging from 95.5% to 98.3% of the nominal concentrations; analytical precision was demonstrated at each dose concentration with relative standard deviation values ≤ 3.1%. The accuracy and homogeneity of these test mixes were confirmed. No test chemical was detected in the blank sample (detection limit was 0.39 mg/mL).

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: **Padimate O**

Task Type: **Formulation Analysis**

RTI Task Number: **0212839.200.003.076**

Chem Task Number: **CHEM11719**

This study/task was audited by the Regulatory and Quality Assurance (RQA) – Quality Assurance Unit and the results of the inspections and audits were reported to the task leader/study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Process Inspection - Formulation Analysis	03/27/2012	03/28/2012
Data and Report Audit	06/12/2012	06/12/2012

Prepared by:

[redacted]
Quality Assurance Specialist

7/17/2012
Date

Reviewed by:

[redacted]
Quality Assurance Specialist

7-17-2012
Date

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Figures

PADIMATE O

1.0 INTRODUCTION

The purpose of this work was to determine the padimate O concentration in corn oil formulation samples submitted by the study lab. To accomplish this objective, a formulation analysis was performed.

2.0 SAMPLE IDENTIFICATION

The following samples were received at RTI analytical laboratory on March 23, 2012, and analyzed for padimate O.

RTI Log Nos.	Target Conc. (mg/mL)	Sample ID	Expiration Date
032312-C-01	200	12-26-1T	May 4, 2012
032312-C-02	200	12-26-1M	May 4, 2012
032312-C-03	200	12-26-1B	May 4, 2012
032312-C-04	64	12-26-2T	May 4, 2012
032312-C-05	64	12-26-2M	May 4, 2012
032312-C-06	64	12-26-2B	May 4, 2012
032312-C-07	20	12-26-3T	May 4, 2012
032312-C-08	20	12-26-3M	May 4, 2012
032312-C-09	20	12-26-3B	May 4, 2012
032312-C-10	0	N135-11-121-32212	May 4, 2012

3.0 SAMPLE ANALYSIS

The methodology used for determining the dose formulations is described in the RTI International report "2-Ethylhexyl-p-dimethylaminobenzoate (Padimate O) in Corn Oil, Dose Formulation Development", (CHEM11137), October 25, 2011. A summary of the method is attached as an appendix to this report.

4.0 SAMPLE RESULTS

The concentrations of padimate O found in the dose formulations and homogeneity results are provided below. Found concentrations are reported in units of mg/mL, and percent recovery (versus the nominal concentration) was calculated using these values.

RTI Log No	Nominal Conc. (mg/mL)	Found Conc.[a] (mg/mL)	Mean Found Conc. (n=3) (mg/mL)	Mean Found/ Nominal	Mean Found Conc. (n=9) (mg/mL)	Mean Found/ Nominal
032312-C-01-1[b]	200	184	186	93.0%		
032312-C-01-2	(top)	195	(4.1% RSD)			
032312-C-01-3		180				
032312-C-02-1	200	190	192	95.9%	191	95.5%
032312-C-02-2	(middle)	190	(1.5% RSD)		(3.1% RSD)	
032312-C-02-3		195				
032312-C-03-1	200	198	195	97.5%		
032312-C-03-2	(bottom)	193	(1.4% RSD)			
032312-C-03-3		194				
032312-C-04-1[b]	64	61.6	61.5	96.0%		
032312-C-04-2	(top)	59.5	(3.0% RSD)			
032312-C-04-3		63.2				
032312-C-05-1	64	61.9	62.3	97.3%	62.9	98.3%
032312-C-05-2	(middle)	62.5	(0.6% RSD)		(3.1% RSD)	
032312-C-05-3		62.5				
032312-C-06-1	64	64.1	65.0	102%		
032312-C-06-2	(bottom)	65.0	(1.3% RSD)			
032312-C-06-3		65.9				
032312-C-07-1[b]	20	19.3	18.9	94.5%		
032312-C-07-2	(top)	18.6	(2.0% RSD)			
032312-C-07-3		18.8				
032312-C-08-1	20	20.4	19.7	98.5%	19.5	97.3%
032312-C-08-2	(middle)	19.3	(3.2% RSD)		(3.0% RSD)	
032312-C-08-3		19.3				
032312-C-09-1	20	20.0	19.8	99.0%		
032312-C-09-2	(bottom)	20.0	(1.4% RSD)			
032312-C-09-3		19.5				
032312-C-10-1[b]	0	ND[c]	NA	NA	NA	NA
032312-C-10-2						
032312-C-10-3						

[a]Quantitation was based on the weighted ($1/x^2$) linear regression equation: $y = 0.5739x - 0.1113$, $r = 0.9998$.

[b]Sample numerical suffixes (1, 2, 3) indicate RTI analytical aliquots.

[c]ND = Not detected; Limit of detection (LOD) = 0.39 mg/mL; Limit of quantitation (LOQ) = 1.3 mg/mL.

Note: Value have been rounded to the appropriate number of significant figures after their performing all calculations in order to minimize round-off error. Some summary parameters

presented in the table may not be accurately reproduced using the rounded values presented elsewhere in the table.

Based on these results, it appears that the mixes are homogeneous and acceptable for use as their average percent found concentrations were within 95.5% and 98.3% of their nominal concentrations and acceptable analytical precision was demonstrated with percent relative standard deviations less than or equal to 3.1%. The two quality control (QC) standards prepared at equivalent concentrations of VA3 (12.0 mg/mL) and VB1 (180 mg/mL) had relative errors of -10% and -7.2% respectively, demonstrating acceptable analytical control.

Representative chromatograms are shown in Figure 1. The vehicle standards plot is illustrated in Figure 2 for the weighted ($1/x^2$) linear regression equations $y = 0.5739x - 0.1113$, $r = 0.9998$.

5.0 ACKNOWLEDGMENT

Personnel contributing to the performance of this task included: [redacted]

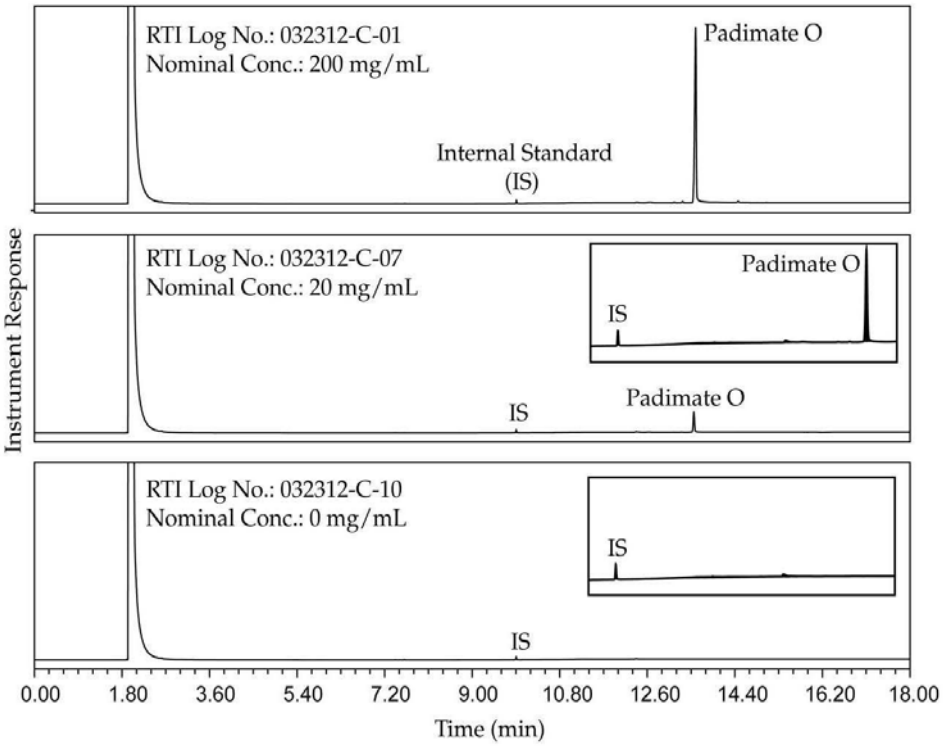

Figure 1. Representative Gas Chromatograms of Padimate O in Corn Oil

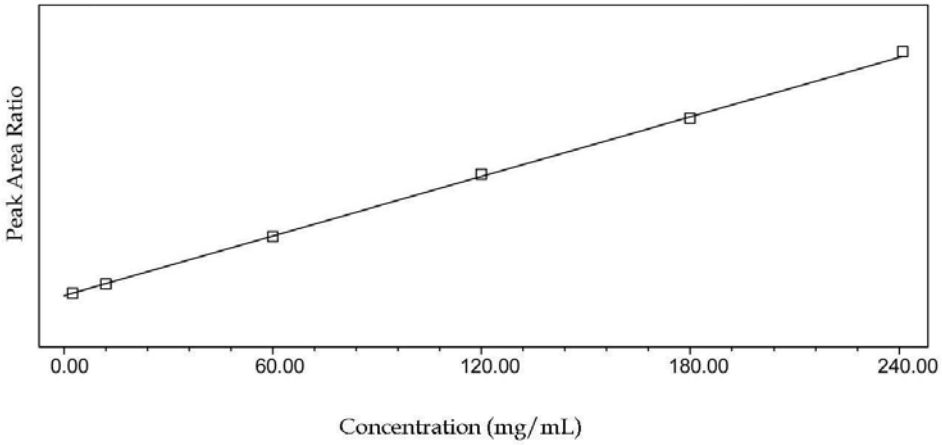

Figure 2. Plot of Vehicle Standards Data – Padimate O in Corn Oil

APPENDIX

Method Summary

Determination of Padimate O in Corn Oil

This appendix summarizes the method used to prepare formulation samples of padimate O in corn oil for analysis, and describes the gas chromatography method.

Preparation of the Internal Standard

An internal standard (IS) stock solution was prepared by transferring 258.11 mg of octanophenone into a 100-mL volumetric flask and diluting to volume with methylene chloride. The IS stock (2.58 mg/mL) was mixed by inversion and transferred to an amber bottle for use and refrigerator storage.

A working IS solution was prepared by transferring 1.0 mL of the IS stock solution to a 100-mL volumetric flask, diluting to volume with methylene chloride, and then mixing by inversion. The working IS solution (0.0258 mg/mL) was transferred to an amber bottle for refrigerator storage.

Preparation of Vehicle Stock Standards

Two vehicle stock standards (VA and VB) were prepared by weighing out aliquots of padimate O and dissolving them in 25 mL of the corn oil vehicle.

VA was prepared with ~7.5 g of padimate O and had a final concentration of 301 mg/mL. VB was prepared from ~ 6.0 g of padimate O and had a final concentration of 240 mg/mL.

Preparation of Vehicle and QC Standards

The standards were prepared by diluting the spiking solutions in corn oil vehicle as described in the table below. The vehicle standards were mixed by inversion. Two additional vehicle standards were prepared as quality control (QC) standards at the VB1 and VA3 concentrations.

Vehicle Standards

Vehicle Std ID	Spiking Solution	Spike Volume (mL)	Final Volume (mL)	Nominal Vehicle Std Conc. (mg/mL)	Actual Vehicle Std Conc.[a] (mg/mL)
VA1	VA	4.0	5	240	241
VB1	VB	3.75	5	180	180
VA2	VA	2.0	5	120	120
VB2	VB	2.5	10	60	60
VA3	VA	1.0	25	12.0	12.0
VB3	VB	0.5	50	2.40	2.40

[a]Example Calculation, VA1: 301 mg/mL x 4.0 mL/5.0 mL = 241 mg/mL.

For each vehicle standard, blank (unspiked corn oil was used for the vehicle blank) and QC standard, 1.0 mL was transferred to a 50-mL volumetric flask and diluted to volume with methylene chloride and mixed by inversion. One milliliter of this primary dilution was transferred to a scintillation vial and 1 mL of the WIS was added, and the sample mixed by inversion. An aliquot was transferred to an autosampler vial for analysis.

Preparation of Formulations Sample for Analysis

Three 1-mL aliquots of each dose formulation sample were transferred to three separate 50-mL volumetric flasks and diluted to volume with methylene chloride, and mixed by inversion. One milliliter of each primary dilution was transferred to a scintillation vial and 1 mL of the WIS was added, and the sample mixed by inversion. An aliquot was transferred to an autosampler vial for analysis.

GC Analysis

Instrument	Agilent 6890N
Column	Phenomenex ZB-5MS (30 m x 0.25 mmID, 0.50 µm film) with 5 m pre-guard
Data System	Empower 2; Build 2154
Inlet Temperature	250 °C
Column Program	70 °C for 1 min., ramp to 270 °C at 20 °C/min., hold for 7 min.
Column Flow	Helium ~1.5 mL/min
Injection Mode	Split ~20:1
Injection Volume	1 µL
Detector: Gas flows	FID at 290 °C: H_2 at 30 mL/min, air at 300 mL/min, N_2 make-up at 30 mL/min

For each dose formulation, the peak area ratio (normalized if required) of each aliquot was calculated (sample area ÷ IS peak area). The found concentration of the analyte was calculated using the peak area ratios and the linear regression equation (weighted $1/x^2$). A mean found concentration was determined for each sample, and for all nine samples at each concentration.

Acceptance criteria for each formulation were a final found concentrations within +/- 10% of the nominal concentration, and a precision (expressed as relative standard deviation for the triplicate preparations) of ≤ 10%.

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NTP Analytical Chemistry Services

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[redacted]
Program Information Coordinator

Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.077
ChemTask No. CHEM11720
CAS No. 118-56-9

HOMOSALATE

IN CORN OIL

FORMULATION ANALYSIS

Mix Dates: March 22, 2012

July 13, 2012

Prepared by:

[redacted] 07-13-12
Task Leader Date

Approved by:

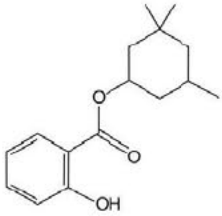
[redacted] 7/13/12
Reshan Fernando, Ph.D. Date
Principal Investigator

Submitted to:

[redacted]
National Institute of Environmental Health Sciences
P.O. Box 12233
111 T. W. Alexander Drive
Research Triangle Park, NC 27709-2233

HOMOSALATE

CAS No.: 118-56-9	Samples Received: 9 x 30 mL and 1 x 100 mL
ChemTask No.: CHEM11720	Dose Formulation Concentrations (RTI Log Nos.): 032312-A-01 to -03: 200 mg/mL; 032312-A-04 to -06: 64 mg/mL: 032312-A-07 to -09: 20 mg/mL; , 032312-A-10: 0 mg/mL
RTI Chemical ID Code: N67	
Program Supported: TOX	
Analysis Dates: Mar 26-28, 2012	Sample Receipt Date: Mar 23, 2012
Interim Results Date: Mar 29, 2012	Submitter: ILS
Mix Dates: Mar 22, 2012	Study Lab (Investigator): ILS [redacted]
Lot No. (Vendor):YT0976 (Spectrum)	Sample Containers: Amber glass bottles
Vendor Purity: 99.88% (Spectrum COA)	Receipt Condition: No damage noted
Vehicle: Corn oil	Storage Condition: Room temperature
Vehicle Lot No.: (Vendor): unknown	

STRUCTURE	MOL. WT.	MOL. FORMULA
	262.34	$C_{16}H_{22}O_3$

EXECUTIVE SUMMARY

In support of the Toxicity Testing Program, a formulation analysis was performed to determine the homosalate content and homogeneity (top, middle and bottom sampling locations) of dose formulations and one vehicle blank prepared in corn oil, submitted by the study lab. Each formulation sample was prepared and analyzed in triplicate.

Analyses conducted using a GC/FID method yielded results ranging from 95.8% to 96.8% of the nominal concentrations; analytical precision was demonstrated at each dose concentration with relative standard deviation values ≤ 3.5%. The accuracy of these test mixes were confirmed. No test chemical was detected in the blank sample (estimated detection limit was 0.09 mg/mL).

In addition these results confirm the homogeneity of each dose formulation over the three sampling locations (top, middle and bottom).

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: **Homosolate**

Task Type: **Formulation Analysis**

RTI Task Number: **0212839.200.003.077**

Chem Task Number: **CHEM11720**

This study/task was audited by the Regulatory and Quality Assurance (RQA) – Quality Assurance Unit and the results of the inspections and audits were reported to the task leader/study director and management as identified below. To the best of our knowledge, the reported results accurately describe the study methods and procedures used, and the reported results accurately reflect the raw data.

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Process Inspection - Formulation Analysis	03/27/2012	03/29/2012
Data and Report Audit	06/13/2012	06/13/2012

Prepared by:

[redacted]
Quality Assurance Specialist

7/13/2012
Date

Reviewed by:

[redacted]
Quality Assurance Specialist

7/13/2012
Date

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Figures

HOMOSALATE

1.0 INTRODUCTION

The purpose of this work was to determine the homosalate content and assess homogeneity of corn oil formulation submitted by the study lab. To accomplish this objective, a formulation analysis was performed.

2.0 SAMPLE IDENTIFICATION

The following samples were received at RTI analytical laboratory on March 23, 2012, and analyzed for homosalate.

RTI Log Nos.	Target Conc. (mg/mL)	Sample ID	Expiration Date
032312-A-01	200	12-24-1T	May 4, 2012
032312-A-02	200	12-24-1M	May 4, 2012
032312-A-03	200	12-24-1B	May 4, 2012
032312-A-04	64	12-24-2T	May 4, 2012
032312-A-05	64	12-24-2M	May 4, 2012
032312-A-06	64	12-24-2B	May 4, 2012
032312-A-07	20	12-24-3T	May 4, 2012
032312-A-08	20	12-24-3M	May 4, 2012
032312-A-09	20	12-24-3B	May 4, 2012
032312-A-10	0	N135-11-121-32212	May 4, 2012

3.0 SAMPLE ANALYSIS

The methodology used for determining the dose formulations is described in the RTI International report "Homosalate in Corn Oil, Dose Formulation Development", (CHEM11139), January 10, 2012. A summary of the method is attached as an appendix to this report.

4.0 SAMPLE RESULTS

The concentrations of homosalate found in the dose formulations are tabulated below. Found concentrations are calculated using the summed area of the two homosalate peaks, reported in units of mg/mL; percent recovery (versus the nominal concentration) was calculated using these values.

RTI Log No.	Nominal Conc. (mg/mL) (Sampling Location)	Found Conc.[a] (mg/mL)	Mean Found Conc. (n=3) (mg/mL)	Mean Found/ Nominal	Mean Found Conc. (n=9) (mg/mL)	Mean Found/ Nominal
032312-A-01-1[b] 032312-A-01-2 032312-A-01-3	200 (top)	190 192 211	197 (5.9% RSD)	98.7%	194 (3.5% RSD)	96.8%
032312-A-02-1 032312-A-02-2 032312-A-02-3	200 (middle)	194 190 190	191 (1.2% RSD)	95.6%		
032312-A-03-1 032312-A-03-2 032312-A-03-3	200 (bottom)	193 190 193	192 (0.9% RSD)	96.0%		
032312-A-04-1[b] 032312-A-04-2 032312-A-04-3	64 (top)	60.8 63.1 62.9	62.3 (2.0% RSD)	97.3%	61.4 (1.8%)	95.9%
032312-A-05-1 032312-A-05-2 032312-A-05-3	64 (middle)	62.0 60.2 60.7	60.9 (1.6% RSD)	95.2%		
032312-A-06-1 032312-A-06-2 032312-A-06-3	64 (bottom)	60.2 61.2 61.3	60.9 (1.0% RSD)	95.2%		
032312-A-07-1[b] 032312-A-07-2 032312-A-07-3	20 (top)	19.6 19.1 19.2	19.3 (1.2% RSD)	96.6%	19.2 (1.4%)	95.8%
032312-A-08-1 032312-A-08-2 032312-A-08-3	20 (middle)	19.1 19.0 19.3	19.1 (0.7% RSD)	95.7%		
032312-A-09-1 032312-A-09-2 032312-A-09-3	20 (bottom)	19.0 18.7 19.5	19.1 (2.3% RSD)	95.3%		
032312-A-10-1[b] 032312-A-10-2 032312-A-10-3	0	ND[c]	NA	NA	NA	NA

[a] Quantitation was based on the weighted ($1/x^2$) linear regression equation: y = 0.6364x - 0.1406, r = 0.9999.

[b] Sample suffixes (1, 2, 3) indicates RTI analytical aliquots.

[c] ND = Not detected; Limit of detection (LOD) = 0.09 mg/mL; Limit of quantitation (LOQ) = 0.3 mg/mL.

Based on these results, it appears that the mixes are acceptable for use as their average percent found concentrations were within 95.8% and 96.8% of their nominal concentrations and acceptable analytical precision was also demonstrated with percent relative standard deviations less than or equal to 3.5%. In addition these results confirm the homogeneity of the three dose formulations. The two quality control (QC) standards prepared at equivalent concentrations of VA3 (12.0 mg/mL) and VB1 (180 mg/mL) had percent relative errors of -3.3 and -4.7 respectively, demonstrating acceptable analytical control.

Representative chromatograms are shown in Figure 1. The vehicle standards plot is illustrated in Figure 2 for the weighted ($1/x^2$) linear regression equations y = 0.6364x - 0.1406, r=0.9999.

5.0 ACKNOWLEDGMENT

Personnel contributing to the performance of this task included: [redacted]

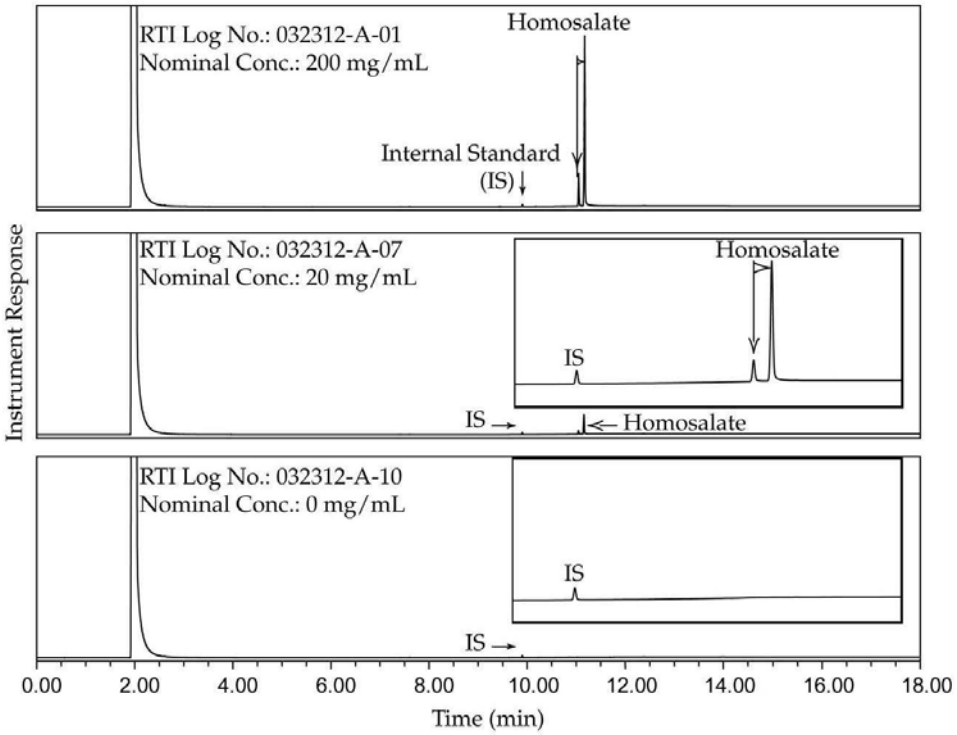

Figure 1. Representative Gas Chromatograms of Homosalate in Corn Oil

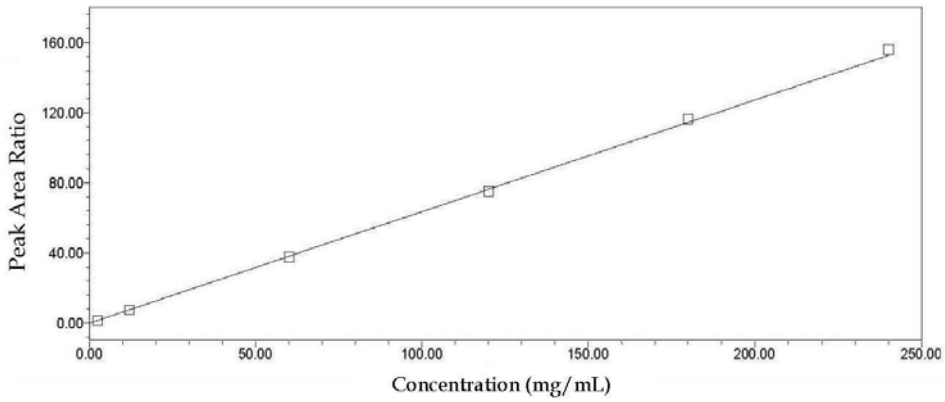

Figure 2. Plot of Vehicle Standards Data – Homosalate in Corn Oil

APPENDIX

Method Summary

Determination of Homosalate in Corn Oil

This appendix summarizes the method used to prepare formulation samples of homosalate in corn oil for analysis, and describes the gas chromatography method.

Preparation of the Internal Standard

An internal standard (IS) stock solution was prepared by transferring 258.11 mg of octanophenone into a 100-mL volumetric flask and diluting to volume with methylene chloride. The IS stock (2.58 mg/mL) was mixed by inversion.

A working IS solution was prepared by transferring 1.0 mL of the IS stock solution to a 100-mL volumetric flask, diluting to volume with methylene chloride, and then mixing by inversion. The working IS solution (0.0258 mg/mL) was transferred to an amber bottle for use.

Preparation of Vehicle Stock Standards

Two vehicle stock standards (VA and VB) were prepared by weighing out aliquots of homosalate and dissolving them in 25 mL of the corn oil vehicle.

VA was prepared with 7.50973 g of homosalate and had a final concentration of 300 mg/mL. VB was prepared from 6.00431 g of homosalate and had a final concentration of 240 mg/mL.

Preparation of Vehicle and QC Standards

The standards were prepared by diluting the spiking solutions in corn oil vehicle as described in the table below. The vehicle standards were mixed by inversion. Two additional vehicle standards were prepared as quality control (QC) standards at the VB1 and VA3 concentrations.

Vehicle Standards

Vehicle Std ID	Spiking Solution	Spike Volume (mL)	Final Volume (mL)	Nominal Vehicle Std Conc. (mg/mL)	Actual Vehicle Std Conc.[a] (mg/mL)
VA1	VA	4.0	5	240	240
VB1	VB	3.75	5	180	180
VA2	VA	2.0	5	120	120
VB2	VB	2.5	10	60	60
VA3	VA	1.0	25	12.0	12.0
VB3	VB	0.5	50	2.40	2.40

[a]Example Calculation, VA1: 300 mg/mL x 4.0 mL/5.0 mL = 240 mg/mL.

For each vehicle standard, blank (unspiked corn oil was for the vehicle blank) and QC standard, 1.0 mL was transferred to a 50-mL volumetric flask and diluted to volume with

methylene chloride and mixed by inversion. One milliliter of this primary dilution was transferred to a scintillation vial and 1 mL of the WIS was added, and the sample mixed by inversion. An aliquot was transferred to an autosampler vial for analysis.

Preparation of Formulations Sample for Analysis

Three 1-mL aliquots of each dose formulation sample were transferred to a 50-mL volumetric flask and diluted to volume with methylene chloride, and mixed by inversion. One milliliter of each primary dilution was transferred to a scintillation vial and 1 mL of the WIS was added, and the sample mixed by inversion. An aliquot was transferred to an autosampler vial for analysis.

GC Analysis

Instrument	Agilent 6890N
Column	Phenomenex ZB-5MS (30 m x 0.25 mmID, 0.50 µm film) with 5 m pre-guard
Data System	Empower 2; Build 2154
Inlet Temperature	250 °C
Column Program	70 °C for 1 min., ramp to 270 °C at 20 °C/min., hold for 7 min.
Column Flow	Helium ~1.5 mL/min
Injection Mode	Split ~20:1
Injection Volume	1 µL
Detector Temp	FID 290 °C

For each dose formulation, the peak area of the two homosalate peaks was summed, then a peak area ratio (normalized if required) was calculated (sample area ÷ IS peak area). The found concentration of the analyte was calculated using the peak area ratios and the linear regression equations (weighted $1/x^2$). A mean found concentration was determined for each sampling location (n=3), and for overall homogeneity confirmation of each formulation (n=9).

Acceptance criteria for each formulation were a final found concentrations within +/- 10% of the nominal concentration, and a precision (expressed as percent relative standard deviation for the triplicate preparations) of ≤ 10%.

Appendix III
Dose Times, Volumes, and Dose Administration

Dose Administration

					Day 1 (4/2/12-4/3/12)			Day 2 (4/3/12-4/4/12)			Day 3 (4/4/12-4/5/12)			Day 4 (4/5/12-4/6/12)	
Group No.:	Animal No.:	Test Substance Dose Level	Actual Dose Concentration (mg/mL)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
1	01	Corn Oil Control	0	56	12:43	1.2	0.0	12:35	1.2	0.0	12:51	1.3	0.0	4/5/12 12:30	23:39
1	02		0	56	12:59	1.2	0.0	12:49	1.2	0.0	13:04	1.2	0.0	4/5/12 13:10	24:06
1	03		0	56	13:09	0.9	0.0	13:03	1.0	0.0	13:22	1.0	0.0	4/5/12 13:46	24:24
1	04		0	56	13:18	1.2	0.0	13:13	1.2	0.0	13:38	1.2	0.0	4/5/12 14:18	24:40
1	05		0	57	13:23	1.2	0.0	13:54	1.2	0.0	13:07	1.2	0.0	4/6/12 12:03	22:56
1	06		0	57	13:33	1.3	0.0	14:10	1.3	0.0	13:15	1.3	0.0	4/6/12 12:31	23:16
1	07		0	57	13:45	1.1	0.0	14:27	1.1	0.0	13:24	1.1	0.0	4/6/12 13:01	23:37
1	08		0	57	13:54	1.1	0.0	14:40	1.1	0.0	13:57	1.1	0.0	4/6/12 13:30	23:33

Group No.:	Animal No.:	Test Substance Dose Level	Actual Dose Concentration (mg/mL)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
2	09	Homosalate (320 mg/kg)	61.4	56	12:45	1.1	314.3	12:36	1.1	316.8	12:52	1.1	304.1	4/5/12 12:34	23:42
2	10		61.4	56	13:00	1.1	303.4	12:50	1.1	304.6	13:07	1.1	294.2	4/5/12 13:14	24:07
2	11		61.4	56	13:10	1.2	308.0	13:03	1.2	306.9	13:23	1.2	298.9	4/5/12 13:50	24:27
2	12		61.4	56	13:19	1.3	302.0	13:14	1.3	306.3	13:40	1.3	300.6	4/5/12 14:21	24:41
2	13		61.4	57	13:24	1.2	312.5	13:56	1.2	311.3	13:09	1.2	306.9	4/6/12 12:06	22:57
2	14		61.4	57	13:34	1.3	317.2	14:11	1.2	295.8	13:16	1.3	308.4	4/6/12 12:36	23:20
2	15		61.4	57	13:46	1.1	296.6	14:28	1.1	295.5	13:25	1.1	293.9	4/6/12 13:04	23:39
2	16		61.4	57	13:55	1.0	308.4	14:41	1.0	302.9	13:57	1.0	296.0	4/6/12 13:32	23:35

Group No.:	Animal No.:	Test Substance Dose Level	Actual Dose Concentration (mg/mL)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
3	17	Homosalate (1000 mg/kg)	194	56	12:47	1.0	1002.1	12:39	1.0	972.4	12:53	1.0	992.3	4/5/12 12:37	23:44
3	18		194	56	13:01	1.2	996.1	12:52	1.2	962.4	13:08	1.2	997.9	4/5/12 13:18	24:10
3	19		194	56	13:10	1.1	976.2	13:05	1.1	971.8	13:24	1.1	983.4	4/5/12 13:52	24:28
3	20		194	56	13:20	1.1	963.4	13:15	1.1	940.9	13:41	1.1	933.9	4/5/12 14:24	24:43
3	21		194	57	13:25	1.2	942.9	13:57	1.2	935.3	13:09	1.2	944.4	4/6/12 12:09	23:00
3	22		194	57	13:35	1.2	987.7	14:12	1.2	1010.9	13:16	1.1	967.4	4/6/12 12:38	23:22
3	23		194	57	13:47	1.3	974.9	14:29	1.2	938.7	13:26	1.2	984.8	4/6/12 13:07	23:41
3	24		194	57	13:56	1.0	984.3	14:42	1.0	984.3	13:58	1.0	976.8	4/6/12 13:37	23:39

Dose Administration

Group No.:	Animal No.:	Test Substance Dose Level	Actual Dose Concentration (mg/mL)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
4	25	Padimate-O (320 mg/kg)	62.9	56	12:49	1.1	305.5	12:40	1.1	303.3	12:55	1.2	320.1	4/5/12 12:44	23:49
4	26		62.9	56	13:02	1.2	327.7	12:53	1.2	317.9	13:12	1.2	304.0	4/5/12 13:22	24:10
4	27		62.9	56	13:12	1.0	326.1	13:05	1.0	314.2	13:28	1.0	305.0	4/5/12 13:56	24:28
4	28		62.9	56	13:21	1.3	320.8	13:16	1.3	321.5	13:42	1.3	312.0	4/5/12 14:26	24:44
4	29		62.9	57	13:25	1.1	309.2	13:58	1.1	307.9	13:10	1.2	327.0	4/6/12 12:11	23:01
4	30		62.9	57	13:36	1.3	324.5	14:14	1.3	325.4	13:17	1.3	317.1	4/6/12 12:40	23:23
4	31		62.9	57	13:47	1.2	327.0	14:30	1.2	326.5	13:27	1.2	312.0	4/6/12 13:09	23:42
4	32		62.9	57	13:57	1.1	321.2	14:43	1.1	319.9	13:59	1.1	307.8	4/6/12 13:39	23:40

Group No.:	Animal No.:	Test Substance Dose Level	Actual Dose Concentration (mg/mL)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
5	33	Padimate-O (1000 mg/kg)	191	56	12:51	1.0	918.7	12:41	1.0	915.2	12:56	1.0	923.2	4/5/12 12:48	23:52
5	34		191	56	13:03	1.1	920.3	12:55	1.1	930.9	13:13	1.1	917.1	4/5/12 13:26	24:13
5	35		191	56	13:13	1.2	919.4	13:07	1.3	980.3	13:29	1.3	975.3	4/5/12 13:59	24:30
5	36		191	56	13:22	1.1	968.2	13:17	1.1	960.7	13:44	1.0	917.8	4/5/12 14:30	24:46
5	37		191	57	13:26	1.3	965.0	14:01	1.3	948.4	13:11	1.3	948.8	4/6/12 12:14	23:03
5	38		191	57	13:37	1.0	911.3	14:16	1.0	924.5	13:19	1.0	939.5	4/6/12 12:43	23:24
5	39		191	57	13:49	1.2	977.8	14:32	1.2	962.6	13:32	1.2	971.6	4/6/12 13:12	23:40
5	40		191	57	13:58	1.3	992.8	14:46	1.2	924.2	14:00	1.3	992.8	4/6/12 13:44	23:44

Group No.:	Animal No.:	Test Substance Dose Level	Actual Dose Concentration (mg/mL)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
6	41	Avobenzone (320 mg/kg)	58.91	56	12:53	1.2	283.7	12:43	1.2	287.5	12:57	1.2	285.6	4/5/12 12:51	23:54
6	42		58.91	56	13:04	1.3	300.1	12:56	1.3	294.8	13:15	1.3	295.5	4/5/12 13:29	24:14
6	43		58.91	56	13:14	1.2	298.0	13:08	1.2	294.9	13:32	1.2	291.4	4/5/12 14:02	24:30
6	44		58.91	56	13:23	1.1	297.0	13:18	1.1	295.9	13:45	1.1	287.0	4/5/12 14:33	24:48
6	45		58.91	57	13:27	1.1	296.4	14:02	1.1	300.8	13:12	1.1	293.5	4/6/12 12:18	23:06
6	46		58.91	57	13:38	1.1	285.1	14:17	1.1	283.0	13:20	1.2	302.0	4/6/12 12:46	23:26
6	47		58.91	57	13:49	1.2	294.8	14:33	1.2	294.4	13:53	1.2	285.2	4/6/12 13:15	23:22
6	48		58.91	57	14:00	1.0	284.2	14:47	1.0	286.7	14:00	1.0	283.2	4/6/12 13:45	23:45

Dose Administration

Group No.:	Animal No.:	Test Substance Dose Level	Actual Dose Concentration (mg/mL)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
7	49	Avobenzone (1000 mg/kg)	209.49	56	12:54	1.2	1081.2	12:44	1.2	1076.6	12:58	1.2	1060.3	4/5/12 12:54	23:56
7	50		209.49	56	13:05	1.1	1023.7	12:57	1.1	1024.6	13:16	1.1	1007.2	4/5/12 13:32	24:16
7	51		209.49	56	13:14	1.1	1015.6	13:09	1.1	1032.0	13:34	1.1	1003.7	4/5/12 14:06	24:32
7	52		209.49	56	13:24	1.2	1076.1	13:19	1.2	1075.7	13:46	1.2	1041.8	4/5/12 14:36	24:50
7	53		209.49	57	13:27	1.2	1008.4	14:05	1.2	1011.6	13:12	1.2	1042.2	4/6/12 12:21	23:09
7	54		209.49	57	13:38	1.0	999.0	14:19	1.0	1005.7	13:21	1.0	1027.4	4/6/12 12:48	23:27
7	55		209.49	57	13:50	1.1	1059.5	14:34	1.1	1056.6	13:54	1.1	1032.9	4/6/12 13:19	23:25
7	56		209.49	57	14:00	1.3	1056.8	14:48	1.3	1047.9	14:01	1.3	1044.2	4/6/12 13:51	23:50

Group No.:	Animal No.:	Test Substance Dose Level	Actual Dose Concentration (mg/mL)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
8	57	Ensulizole (320 mg/kg)	56.6	56	12:55	1.2	272.6	12:45	1.3	290.1	12:59	1.3	279.3	4/5/12 12:58	23:59
8	58		56.6	56	13:06	1.2	281.2	12:58	1.2	278.1	13:17	1.3	290.5	4/5/12 13:35	24:18
8	59		56.6	56	13:15	1.1	295.9	13:10	1.1	293.7	13:35	1.1	288.8	4/5/12 14:09	24:34
8	60		56.6	56	13:25	1.1	282.5	13:20	1.1	275.9	13:47	1.2	294.8	4/5/12 14:40	24:53
8	61		56.6	57	13:30	1.0	277.7	14:07	1.1	294.0	13:13	1.1	285.1	4/6/12 12:23	23:10
8	62		56.6	57	13:39	1.1	270.9	14:20	1.2	291.9	13:21	1.2	292.4	4/6/12 12:50	23:29
8	63		56.6	57	13:51	1.2	291.0	14:36	1.2	283.2	13:55	1.2	278.4	4/6/12 13:21	23:26
8	64		56.6	57	14:01	1.2	280.2	14:49	1.2	275.1	14:04	1.3	289.7	4/6/12 13:52	23:48

Group No.:	Animal No.:	Test Substance Dose Level	Actual Dose Concentration (mg/mL)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
9	65	Ensulizole (1000 mg/kg)	189	56	12:56	1.2	922.0	12:46	1.3	979.7	13:01	1.3	953.1	4/5/12 13:02	24:01
9	66		189	56	13:07	1.1	924.8	12:59	1.1	911.4	13:18	1.2	961.0	4/5/12 13:38	24:20
9	67		189	56	13:16	1.0	919.7	13:11	1.0	905.6	13:36	1.1	976.5	4/5/12 14:12	24:36
9	68		189	56	13:26	1.0	931.5	13:21	1.0	922.0	13:48	1.1	985.3	4/5/12 14:43	24:55
9	69		189	57	13:31	1.1	911.8	14:08	1.2	971.7	13:13	1.2	949.0	4/6/12 12:26	23:13
9	70		189	57	13:40	1.2	917.1	14:21	1.2	913.0	13:22	1.3	959.4	4/6/12 12:53	23:31
9	71		189	57	13:52	1.2	947.0	14:37	1.2	949.7	13:55	1.2	931.4	4/6/12 13:24	23:29
9	72		189	57	14:02	1.3	959.0	14:50	1.3	930.0	14:05	1.4	976.4	4/6/12 13:55	23:50

				Day 1 (4/2/12-4/3/12)		Day 2 (4/3/12-4/4/12)		Day 3 (4/4/12-4/5/12)		Day 4 (4/5/12-4/6/12)	
Group No.:	Animal No.:	Test Substance Dose Level	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Time of Death	Time From Last Administration
10	73	17α -Ethinyl estradiol (0.05 mg/kg)	56	12:58	1.2	12:47	1.2	13:03	1.2	4/5/12 13:06	24:03
10	74		56	13:08	1.0	13:01	1.1	13:20	1.0	4/5/12 13:41	24:21
10	75		56	13:17	1.2	13:12	1.3	13:37	1.3	4/5/12 14:15	24:38
10	76		56	13:27	1.1	13:22	1.1	13:51	1.1	4/5/12 14:45	24:54
10	77		57	13:32	1.1	14:09	1.1	13:14	1.1	4/6/12 12:29	23:15
10	78		57	13:43	1.3	14:26	1.3	13:23	1.2	4/6/12 12:56	23:33
10	79		57	13:52	1.1	14:39	1.1	13:56	1.1	4/6/12 13:26	23:30
10	80		57	14:04	1.2	14:52	1.2	14:05	1.2	4/6/12 13:57	23:52

Appendix IV
Clinical Observation Data

Clinical Observations

				Day 1 (4/2/1-4/3/12)	Day 2 (4/3/12-4/4/12)	Day 3 (4/4/12-4/5/12)	Day 4 (4/5/12-4/6/12)
Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**
1	01	F	Corn Oil Control	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
1	02	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
1	03	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
1	04	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
1	05	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
1	06	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
1	07	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
1	08	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back

Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**
2	09	F	Homosalate (320 mg/kg)	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
2	10	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
2	11	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
2	12	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
2	13	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
2	14	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
2	15	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
2	16	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back

Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**
3	17	F	Homosalate (1000 mg/kg)	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
3	18	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
3	19	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
3	20	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
3	21	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
3	22	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
3	23	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
3	24	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back

Clinical Observations

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
4	25	F	Padimate-O (320 mg/kg)	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
4	26	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
4	27	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
4	28	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
4	29	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
4	30	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
4	31	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
4	32	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
5	33	F	Padimate-O (1000 mg/kg)	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
5	34	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
5	35	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
5	36	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
5	37	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
5	38	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
5	39	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
5	40	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
6	41	F	Avobenzone (320 mg/kg)	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
6	42	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
6	43	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
6	44	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
6	45	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
6	46	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
6	47	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
6	48	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back

Clinical Observations

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
7	49	F	Avobenzone (1000 mg/kg)	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
7	50	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
7	51	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
7	52	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
7	53	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
7	54	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
7	55	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
7	56	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
8	57	F	Ensulizole (320 mg/kg)	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
8	58	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
8	59	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
8	60	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
8	61	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
8	62	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
8	63	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
8	64	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
9	65	F	Ensulizole (1000 mg/kg)	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
9	66	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
9	67	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
9	68	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
9	69	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
9	70	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
9	71	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
9	72	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
10	73	F	17α -Ethinyl estradiol (0.05 mg/kg)	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
10	74	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
10	75	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
10	76	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
10	77	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
10	78	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
10	79	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back
10	80	F		Hairless Spot on back	Hairless Spot on back	Hairless Spot on back	Hairless Spot on back

Cage-side Clinical Observations

				Day 1 (4/2/12-4/3/12)	Day 2 (4/3/12-4/4/12)	Day 3 (4/4/12-4/5/12)
Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Cage-Side Observation**	**Cage-Side Observation**	**Cage-Side Observation**
1	01	F	Corn Oil Control	Normal	Normal	Normal
1	02	F		Normal	Normal	Normal
1	03	F		Normal	Normal	Normal
1	04	F		Normal	Normal	Normal
1	05	F		Normal	Normal	Normal
1	06	F		Normal	Normal	Normal
1	07	F		Normal	Normal	Normal
1	08	F		Normal	Normal	Normal

Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Cage-Side Observation**	**Cage-Side Observation**	**Cage-Side Observation**
2	09	F	Homosalate (320 mg/kg)	Normal	Normal	Normal
2	10	F		Normal	Normal	Normal
2	11	F		Normal	Normal	Normal
2	12	F		Normal	Normal	Normal
2	13	F		Normal	Normal	Normal
2	14	F		Normal	Normal	Normal
2	15	F		Normal	Normal	Normal
2	16	F		Normal	Normal	Normal

Cage-side Clinical Observations

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Cage-Side Observation	Cage-Side Observation	Cage-Side Observation
3	17	F	Homosalate (1000 mg/kg)	Normal	Normal	Normal
3	18	F		Normal	Normal	Normal
3	19	F		Normal	Normal	Normal
3	20	F		Normal	Normal	Normal
3	21	F		Normal	Normal	Normal
3	22	F		Normal	Normal	Normal
3	23	F		Normal	Normal	Normal
3	24	F		Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Cage-Side Observation	Cage-Side Observation	Cage-Side Observation
4	25	F	Padimate-O (320 mg/kg)	Normal	Normal	Normal
4	26	F		Normal	Normal	Normal
4	27	F		Normal	Normal	Normal
4	28	F		Normal	Normal	Normal
4	29	F		Normal	Normal	Normal
4	30	F		Normal	Normal	Normal
4	31	F		Normal	Normal	Normal
4	32	F		Normal	Normal	Normal

Cage-side Clinical Observations

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Cage-Side Observation	Cage-Side Observation	Cage-Side Observation
5	33	F	Padimate-O (1000 mg/kg)	Normal	Normal	Normal
5	34	F		Normal	Normal	Normal
5	35	F		Normal	Normal	Normal
5	36	F		Normal	Normal	Normal
5	37	F		Normal	Normal	Normal
5	38	F		Normal	Normal	Normal
5	39	F		Normal	Normal	Normal
5	40	F		Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Cage-Side Observation	Cage-Side Observation	Cage-Side Observation
6	41	F	Avobenzone (320 mg/kg)	Normal	Normal	Normal
6	42	F		Normal	Normal	Normal
6	43	F		Normal	Normal	Normal
6	44	F		Normal	Normal	Normal
6	45	F		Normal	Normal	Normal
6	46	F		Normal	Normal	Normal
6	47	F		Normal	Normal	Normal
6	48	F		Normal	Normal	Normal

Cage-side Clinical Observations

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Cage-Side Observation	Cage-Side Observation	Cage-Side Observation
7	49	F	Avobenzone (1000 mg/kg)	Normal	Normal	Normal
7	50	F		Normal	Normal	Normal
7	51	F		Normal	Normal	Normal
7	52	F		Normal	Normal	Normal
7	53	F		Normal	Normal	Normal
7	54	F		Normal	Normal	Normal
7	55	F		Normal	Normal	Normal
7	56	F		Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Cage-Side Observation	Cage-Side Observation	Cage-Side Observation
8	57	F	Ensulizole (320 mg/kg)	Normal	Normal	Normal
8	58	F		Normal	Normal	Normal
8	59	F		Normal	Normal	Normal
8	60	F		Normal	Normal	Normal
8	61	F		Normal	Normal	Normal
8	62	F		Normal	Normal	Normal
8	63	F		Normal	Normal	Normal
8	64	F		Normal	Normal	Normal

Cage-side Clinical Observations

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Cage-Side Observation	Cage-Side Observation	Cage-Side Observation
9	65	F	Ensulizole (1000 mg/kg)	Normal	Normal	Normal
9	66	F		Normal	Normal	Normal
9	67	F		Normal	Normal	Normal
9	68	F		Normal	Normal	Normal
9	69	F		Normal	Normal	Normal
9	70	F		Normal	Normal	Normal
9	71	F		Normal	Normal	Normal
9	72	F		Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Cage-Side Observation	Cage-Side Observation	Cage-Side Observation
10	73	F	17α -Ethinyl estradiol (0.05 mg/kg)	Normal	Normal	Normal
10	74	F		Normal	Normal	Normal
10	75	F		Normal	Normal	Normal
10	76	F		Normal	Normal	Normal
10	77	F		Normal	Normal	Normal
10	78	F		Normal	Normal	Normal
10	79	F		Normal	Normal	Normal
10	80	F		Normal	Normal	Normal

Appendix V
Body Weight Data

Body Weights

				Day 1 (4/2/1-4/3/12)	Day 2 (4/3/12-4/4/12)	Day 3 (4/4/12-4/5/12)	Day 4 (4/5/12-4/6/12)	
Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Terminal Body Weight (g)**	**Body Weight Gain (g)**
1	01	F	Corn Oil Control	243.6	246.6	255.0	261.6	18.0
1	02	F		234.5	242.3	245.6	252.0	17.5
1	03	F		187.9	190.3	195.9	200.5	12.6
1	04	F		231.7	233.4	240.4	246.5	14.8
1	05	F		232.7	239.8	240.5	246.0	13.3
1	06	F		259.1	265.5	269.5	274.8	15.7
1	07	F		223.5	222.0	228.7	242.8	19.3
1	08	F		220.8	221.9	228.5	232.5	11.7
			Mean	**229.2**	**232.7**	**238.0**	**244.6**	**15.4**
			SD	**20.6**	**22.2**	**21.7**	**21.9**	**2.7**
			Count	**8**	**8**	**8**	**8**	**8**

Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Terminal Body Weight (g)**	**Body Weight Gain (g)**
2	09	F	Homosalate (320 mg/kg)	214.9	213.2	222.1	224.5	9.6
2	10	F		222.6	221.7	229.6	231.1	8.5
2	11	F		239.2	240.1	246.5	250.3	11.1
2	12	F		264.3	260.6	265.5	263.9	-0.4
2	13	F		235.8	236.7	240.1	244.1	8.3
2	14	F		251.6	249.1	258.8	262.2	10.6
2	15	F		227.7	228.6	229.8	242.7	15.0
2	16	F		199.1	202.7	207.4	213.0	13.9
			Mean	**231.9**	**231.6**	**237.5**	**241.5**	**9.6**
			SD	**20.6**	**19.0**	**19.2**	**17.8**	**4.7**
			Count	**8**	**8**	**8**	**8**	**8**
			% of Control	**101.2**	**99.5**	**99.8**	**98.7**	

Body Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Terminal Body Weight (g)	Body Weight Gain (g)
3	17	F	Homosalate (1000 mg/kg)	193.6	199.5	195.5	195.1	1.5
3	18	F		233.7	241.9	233.3	236.7	3.0
3	19	F		218.6	219.6	217.0	208.8	-9.8
3	20	F		221.5	226.8	228.5	227.0	5.5
3	21	F		246.9	248.9	246.5	244.3	-2.6
3	22	F		235.7	230.3	220.6	210.5	-25.2
3	23	F		258.7	248.0	236.4	231.7	-27.0
3	24	F		197.1	197.1	198.6	195.5	-1.6
			Mean	**225.7**	**226.5**	**222.1**	**218.7**	**-7.0**
			SD	**22.7**	**20.2**	**17.9**	**18.8**	**12.6**
			Count	**8**	**8**	**8**	**8**	**8**
			% of Control	**98.5**	**97.3**	**93.3**	**89.4**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Terminal Body Weight (g)	Body Weight Gain (g)
4	25	F	Padimate-O (320 mg/kg)	226.5	228.1	235.8	238.4	11.9
4	26	F		230.3	237.4	248.3	249.5	19.2
4	27	F		192.9	200.2	206.2	206.8	13.9
4	28	F		254.9	254.3	262.1	265.3	10.4
4	29	F		223.8	224.7	230.8	234.6	10.8
4	30	F		252.0	251.3	257.9	261.7	9.7
4	31	F		230.8	231.2	241.9	248.9	18.1
4	32	F		215.4	216.3	224.8	230.8	15.4
			Mean	**228.3**	**230.4**	**238.5**	**242.0**	**13.7**
			SD	**19.7**	**17.8**	**18.3**	**18.8**	**3.6**
			Count	**8**	**8**	**8**	**8**	**8**
			% of Control	**99.6**	**99.0**	**100.2**	**98.9**	

Body Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Terminal Body Weight (g)	Body Weight Gain (g)
5	33	F	Padimate-O (1000 mg/kg)	207.9	208.7	206.9	212.6	4.7
5	34	F		228.3	225.7	229.1	242.5	14.2
5	35	F		249.3	253.3	254.6	254.2	4.9
5	36	F		217.0	218.7	208.1	199.4	-17.6
5	37	F		257.3	261.8	261.7	260.4	3.1
5	38	F		209.6	206.6	203.3	208.5	-1.1
5	39	F		234.4	238.1	235.9	233.7	-0.7
5	40	F		250.1	248.0	250.1	255.9	5.8
			Mean	**231.7**	**232.6**	**231.2**	**233.4**	**1.7**
			SD	**19.2**	**20.8**	**23.2**	**23.8**	**9.1**
			Count	**8**	**8**	**8**	**8**	**8**
			% of Control	**101.1**	**100.0**	**97.1**	**95.4**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Terminal Body Weight (g)	Body Weight Gain (g)
6	41	F	Avobenzone (320 mg/kg)	249.2	245.9	247.5	249.5	0.3
6	42	F		255.2	259.8	259.2	264.1	8.9
6	43	F		237.2	239.7	242.6	252.7	15.5
6	44	F		218.2	219.0	225.8	228.9	10.7
6	45	F		218.6	215.4	220.8	226.1	7.5
6	46	F		227.3	229.0	234.1	238.8	11.5
6	47	F		239.8	240.1	247.9	257.2	17.4
6	48	F		207.3	205.5	208.0	215.2	7.9
			Mean	**231.6**	**231.8**	**235.7**	**241.6**	**10.0**
			SD	**16.6**	**17.9**	**16.8**	**17.1**	**5.3**
			Count	**8**	**8**	**8**	**8**	**8**
			% of Control	**101.0**	**99.6**	**99.0**	**98.8**	

Body Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Terminal Body Weight (g)	Body Weight Gain (g)
7	49	F	Avobenzone (1000 mg/kg)	232.5	233.5	237.1	244.4	11.9
7	50	F		225.1	224.9	228.8	232.6	7.5
7	51	F		226.9	223.3	229.6	235.9	9.0
7	52	F		233.6	233.7	241.3	245.3	11.7
7	53	F		249.3	248.5	241.2	253.4	4.1
7	54	F		209.7	208.3	203.9	206.8	-2.9
7	55	F		217.5	218.1	223.1	230.6	13.1
7	56	F		257.7	259.9	260.8	265.3	7.6
			Mean	**231.5**	**231.3**	**233.2**	**239.3**	**7.8**
			SD	**15.8**	**16.6**	**16.5**	**17.4**	**5.2**
			Count	**8**	**8**	**8**	**8**	**8**
			% of Control	**101.0**	**99.4**	**98.0**	**97.8**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Terminal Body Weight (g)	Body Weight Gain (g)
8	57	F	Ensulizole (320 mg/kg)	249.2	253.6	263.4	268.8	19.6
8	58	F		241.5	244.2	253.3	258.7	17.2
8	59	F		210.4	212.0	215.6	221.1	10.7
8	60	F		220.4	225.7	230.4	236.9	16.5
8	61	F		203.8	211.8	218.4	224.5	20.7
8	62	F		229.8	232.7	232.3	246.5	16.7
8	63	F		233.4	239.8	244.0	252.5	19.1
8	64	F		242.4	246.9	254.0	257.3	14.9
			Mean	**228.9**	**233.3**	**238.9**	**245.8**	**16.9**
			SD	**16.1**	**15.7**	**17.5**	**17.0**	**3.1**
			Count	**8**	**8**	**8**	**8**	**8**
			% of Control	**99.8**	**100.3**	**100.4**	**100.5**	

Body Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Terminal Body Weight (g)	Body Weight Gain (g)
9	65	F	Ensulizole (1000 mg/kg)	246.0	250.8	257.8	258.6	12.6
9	66	F		224.8	228.1	236.0	241.4	16.6
9	67	F		205.5	208.7	212.9	219.5	14.0
9	68	F		202.9	205.0	211.0	214.8	11.9
9	69	F		228.0	233.4	239.0	245.3	17.3
9	70	F		247.3	248.4	256.1	256.1	8.8
9	71	F		239.5	238.8	243.5	253.1	13.6
9	72	F		256.2	264.2	271.0	281.0	24.8
			Mean	**231.3**	**234.7**	**240.9**	**246.2**	**15.0**
			SD	**19.6**	**20.5**	**21.2**	**21.5**	**4.8**
			Count	**8**	**8**	**8**	**8**	**8**
			% of Control	**100.9**	**100.8**	**101.2**	**100.7**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Terminal Body Weight (g)	Body Weight Gain (g)
10	73	F	17α -Ethinyl estradiol (0.05 mg/kg)	231.8	231.9	235.5	239.2	7.4
10	74	F		207.6	210.4	208.6	210.0	2.4
10	75	F		247.1	252.9	251.0	252.2	5.1
10	76	F		216.4	216.5	217.9	216.9	0.5
10	77	F		228.5	227.5	222.4	222.8	-5.7
10	78	F		260.2	256.0	248.3	248.9	-11.3
10	79	F		215.1	214.0	212.6	214.8	-0.3
10	80	F		244.3	245.1	239.6	247.4	3.1
			Mean	**231.4**	**231.8**	**229.5**	**231.5**	**0.2**
			SD	**18.2**	**17.9**	**16.3**	**17.2**	**6.1**
			Count	**8**	**8**	**8**	**8**	**8**
			% of Control	**100.9**	**99.6**	**96.4**	**94.7**	

Appendix VI
Tissue Weight Data

Tissue Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Terminal Body Weight (g)	Uterine Weight, wet (g)	Uterine Weight, blotted (g)	Relative Uterine Weight, blotted (g)
1	01	F	Corn Oil Control	261.6	0.0818	0.0741	0.0283
1	02	F		252.0	0.0860	0.0833	0.0331
1	03	F		200.5	0.0817	0.0743	0.0371
1	04	F		246.5	0.0535	0.0460	0.0187
1	05	F		246.0	0.0830	0.0801	0.0326
1	06	F		274.8	0.0712	0.0688	0.0250
1	07	F		242.8	0.0623	0.0552	0.0227
1	08	F		232.5	0.0671	0.0608	0.0262
			Mean	**244.6**	**0.0733**	**0.0678**	**0.0279**
			SD	**21.9**	**0.0117**	**0.0129**	**0.0060**
			CV	**9.0**	**15.9217**	**18.9591**	**21.6222**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Terminal Body Weight (g)	Uterine Weight, wet (g)	Uterine Weight, blotted (g)
2	09	F	Homosalate (320 mg/kg)	224.5	0.0982	0.0868
2	10	F		231.1	0.0697	0.0629
2	11	F		250.3	0.0912	0.0888
2	12	F		263.9	0.0921	0.0891
2	13	F		244.1	0.0794	0.0719
2	14	F		262.2	0.1005	0.0928
2	15	F		242.7	0.0578	0.0553
2	16	F		213.0	0.0643	0.0587
			Mean	**241.5**	**0.0817**	**0.0758**
			SD	**17.8**	**0.0162**	**0.0154**
			CV	**7.4**	**19.8961**	**20.2590**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Terminal Body Weight (g)	Uterine Weight, wet (g)	Uterine Weight, blotted (g)
3	17	F	Homosalate (1000 mg/kg)	195.1	0.0692	0.0662
3	18	F		236.7	0.0754	0.0721
3	19	F		208.8	0.0763	0.0665
3	20	F		227.0	0.0563	0.0483
3	21	F		244.3	0.0664	0.0633
3	22	F		210.5	0.0758	0.0733
3	23	F		231.7	0.0821	0.0744
3	24	F		195.5	0.0483	0.0442
			Mean	**218.7**	**0.0687**	**0.0635**
			SD	**18.8**	**0.0114**	**0.0114**
			CV	**8.6**	**16.5728**	**17.9317**

Tissue Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Terminal Body Weight (g)	Uterine Weight, wet (g)	Uterine Weight, blotted (g)
4	25	F	Padimate-O (320 mg/kg)	238.4	0.0612	0.0552
4	26	F		249.5	0.0871	0.0787
4	27	F		206.8	0.0624	0.0598
4	28	F		265.3	0.0818	0.0779
4	29	F		234.6	0.0690	0.0619
4	30	F		261.7	0.0773	0.0715
4	31	F		248.9	0.0776	0.0762
4	32	F		230.8	0.0679	0.0637
			Mean	**242.0**	**0.0730**	**0.0681**
			SD	**18.8**	**0.0093**	**0.0091**
			CV	**7.8**	**12.7759**	**13.3487**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Terminal Body Weight (g)	Uterine Weight, wet (g)	Uterine Weight, blotted (g)
5	33	F	Padimate-O (1000 mg/kg)	212.6	0.0683	0.0657
5	34	F		242.5	0.0599	0.0571
5	35	F		254.2	0.0755	0.0689
5	36	F		199.4	0.0538	0.0488
5	37	F		260.4	0.0781	0.0725
5	38	F		208.5	0.0588	0.0555
5	39	F		233.7	0.0739	0.0677
5	40	F		255.9	0.0723	0.0664
			Mean	**233.4**	**0.0676**	**0.0628**
			SD	**23.8**	**0.0090**	**0.0081**
			CV	**10.2**	**13.2608**	**12.8815**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Terminal Body Weight (g)	Uterine Weight, wet (g)	Uterine Weight, blotted (g)
6	41	F	Avobenzone (320 mg/kg)	249.5	0.0777	0.0703
6	42	F		264.1	0.0676	0.0622
6	43	F		252.7	0.0876	0.0846
6	44	F		228.9	0.0617	0.0564
6	45	F		226.1	0.0949	0.0871
6	46	F		238.8	0.0851	0.0775
6	47	F		257.2	0.0638	0.0613
6	48	F		215.2	0.0733	0.0694
			Mean	**241.6**	**0.0765**	**0.0711**
			SD	**17.1**	**0.0120**	**0.0112**
			CV	**7.1**	**15.6857**	**15.7165**

Tissue Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Terminal Body Weight (g)	Uterine Weight, wet (g)	Uterine Weight, blotted (g)
7	49	F	Avobenzone (1000 mg/kg)	244.4	0.0769	0.0740
7	50	F		232.6	0.0719	0.0686
7	51	F		235.9	0.0715	0.0634
7	52	F		245.3	0.0653	0.0588
7	53	F		253.4	0.0651	0.0623
7	54	F		206.8	0.0690	0.0646
7	55	F		230.6	0.0659	0.0588
7	56	F		265.3	0.0727	0.0649
			Mean	**239.3**	**0.0698**	**0.0644**
			SD	**17.4**	**0.0042**	**0.0050**
			CV	**7.3**	**6.0384**	**7.8319**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Terminal Body Weight (g)	Uterine Weight, wet (g)	Uterine Weight, blotted (g)
8	57	F	Ensulizole (320 mg/kg)	268.8	0.1012	0.0913
8	58	F		258.7	0.0616	0.0555
8	59	F		221.1	0.0803	0.0775
8	60	F		236.9	0.0677	0.0644
8	61	F		224.5	0.0643	0.0587
8	62	F		246.5	0.0712	0.0642
8	63	F		252.5	0.0844	0.0799
8	64	F		257.3	0.0850	0.0802
			Mean	**245.8**	**0.0770**	**0.0715**
			SD	**17.0**	**0.0133**	**0.0125**
			CV	**6.9**	**17.2506**	**17.5242**

Tissue Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Terminal Body Weight (g)	Uterine Weight, wet (g)	Uterine Weight, blotted (g)
9	65	F	Ensulizole (1000 mg/kg)	258.6	0.0648	0.0614
9	66	F		241.4	0.0923	0.0881
9	67	F		219.5	0.0681	0.0612
9	68	F		214.8	0.0637	0.0571
9	69	F		245.3	0.0750	0.0748
9	70	F		256.1	0.0548	0.0515
9	71	F		253.1	0.0569	0.0507
9	72	F		281.0	0.0837	0.0747
			Mean	**246.2**	**0.0699**	**0.0649**
			SD	**21.5**	**0.0130**	**0.0131**
			CV	**8.7**	**18.5940**	**20.1648**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Terminal Body Weight (g)	Uterine Weight, wet (g)	Uterine Weight, blotted (g)
10	73	F	17α -Ethinyl estradiol (0.05 mg/kg)	239.2	0.2264	0.1714
10	74	F		210.0	0.1160	0.1027
10	75	F		252.2	0.2171	0.1759
10	76	F		216.9	0.2633	0.1870
10	77	F		222.8	0.7658	0.3186
10	78	F		248.9	0.3711	0.2478
10	79	F		214.8	0.1414	0.1295
10	80	F		247.4	0.3552	0.2122
			Mean	**231.5**	**0.3070**	**0.1931**
			SD	**17.2**	**0.2061**	**0.0677**
			CV	**7.4**	**67.1233**	**35.0774**

Appendix VII
Study Protocol

Study Title
The Uterotrophic Assay with
Avobenzone, Ensulizole, Homosalate, and Padimate-O

ILS Project-Study Number
N135-247

Performing Laboratory
Integrated Laboratory Systems, Inc.
635 Keystone Park Drive, Suite 100
Durham, NC 27713

Sponsor
National Institutes of Environmental Health
P.O. Box 12233
Research Triangle Park, NC 27709

Study Protocol Approval

[redacted]

Chief Toxicology Branch
National Toxicology Program, NIEHS

3/25/12
Date

[redacted]

Contract Office Technical Representative
National Toxicology Program, NIEHS

3/26/12
Date

[redacted]

Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

3/26/12
Date

[redacted]

Principal Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

4-2-2012
Date

TABLE OF CONTENTS

INTRODUCTION

1.1 Background

The Endocrine Disruptor Screening Program (EDSP) reflects a two-tiered approach to implement the statutory testing requirements of FFDCA section 408(p) (21 U.S.C. 346a). The U.S. EPA will use the data collected under the EDSP, along with other information to determine if a pesticide chemical, or other substances, may pose a risk to human health or the environment due to disruption of the endocrine system.

EDSP Tier 1 screening assays will be used to identify substances that have the potential to interact with the estrogen, androgen, or thyroid hormone (Test guidelines in the OPPTS 890 series). The determination of a chemicals ability to interact with hormone systems will be made on a weight-of-evidence basis, taking into account data from the Tier 1 assays and other scientifically-relevant information available. If a substance interacts with a hormone system, it does not imply that when used it will cause adverse effects in humans or ecological systems. The Uterotrophic Assay (OPPTS 890.1600) is used to screen substances for estrogenicity and is one of four *in vivo* mammalian assays in the EDSP Tier 1 battery of assays.

1.2 Purpose

The purpose of this assay is to screen four test substances selected by the National Toxicology Program for their potential estrogenicity using the ovariectomized rat model Uterotrophic Assay (OPPTS 890.1600).

1.3 Regulatory Compliance

This study will be conducted in accordance with Good Laboratory Practice regulations as promulgated by the United States Environmental Protection Agency's (U.S. EPA) Good Laboratory Practice (GLP) Regulations (40 CFR Part 160), the Endocrine Disruptor Screening Program Test Guideline OPPTS 890.1600: Uterotrophic Assay (U.S. EPA), and ILS Standard Operating Procedures. The study protocol will be reviewed by the ILS Quality Assurance (QA) Unit before final approval by the Sponsor. All changes to the study protocol will be approved by the Sponsor.

17-α Ethinyl estradiol will not be analyzed as stated in 40 CFR 160.113(a)(1) of the U.S. EPA GLP requirements, a positive response in the test system following 17α-ethinyl estradiol administration will be evident following statistical analysis of the tissue weights.

A QA inspection of critical phases will be conducted to assure the quality and integrity of the study results and conformance to the study protocol. An audit of the final report will be conducted to determine consistency between reported information and raw data. An appropriate QA statement will be included in the final report.

1.4 Sponsor

NIEHS
P.O. Box 12233
Research Triangle Park, NC 27709

[redacted]
Contract Office Technical Representative
National Toxicology Program, National Institutes of Environmental Health

NTP Investigator
[redacted]
Telephone No.:
Facsimile No.:
E-mail:

[redacted]

1.5 Testing Facility
Integrated Laboratory Systems, Inc. (ILS)

Shipping Address:	635 Keystone Park Drive, Suite 100 Durham, NC27713
Mailing Address:	P.O. Box 13501 Research Triangle Park, NC27709

Study Director
[redacted]
Telephone No.:
Facsimile No.:
E-mail:

[redacted]

1.6 Study Dates

Animal Arrival Date:	March 26, 2012
Experimental Start Date:	April 2, 2012
Experimental Termination Date:	April 6, 2012

TEST SUBSTANCE, REFERENCE SUBSTANCE, VEHICLE

2.1 Test Substance: 2-Phenyl-5-benzimidazolesulfonic Acid (Ensulizole)

CAS No.	27503-81-7
Source:	Sigma-Aldrich
Lot/Batch No.:	05117JE
ILS Repository No.:	12-25
Formula:	$C_{13}H_{10}N_2O_3S$

Description:	White Powder
Purity:	99.9%
Dose Formulation:	Corn Oil
Storage:	
Test Substance:	Ambient temperature protected from light
Dose Formulation:	Ambient temperature protected from light
Stability:	
Dose Formulation:	Ensulizole in corn oil stored at ambient temperature was shown to be stable for 43 days (Blake, 2012a).

2.2 Test Substance: Butyl-methoxydibenzoylmethane (Avobenzone)

CAS No.	70356-09-1
Source:	Universal Preserv-A-Chem, Inc.
Lot/Batch No.:	L802809
Expiration:	14 June 2012
ILS Repository No.:	12-19
Formula:	$C_{20}H_{22}O_3$
Description:	Off white to yellowish, crystalline powder
Purity:	98.3%
Dose Formulation:	Corn Oil
Storage:	
Test Substance:	Ambient temperature protected from light
Dose Formulation:	Ambient temperature protected from light

Stability:

Dose Formulation:	Avobenzone in corn oil stored at ambient temperature was shown to be stable for 42 days (Aillon, 2012).

2.3 Test Substance: 3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)

CAS No.	118-56-9
Source:	Spectrum Laboratory Products Inc
Lot/Batch No.:	YT0976
ILS Repository No.:	12-24
Formula:	$C_{16}H_{22}O_3$
Description:	Colorless to light yellow liquid
Purity:	99.88%
Dose Formulation:	Corn Oil
Storage:	
Test Substance:	Ambient temperature
Dose Formulation:	Ambient temperature protected from light
Stability:	
Dose Formulation:	Homosalate in corn oil stored at ambient temperature was shown to be stable for 42 days (Blake, 2012b).

2.4 Test Substance: 2-Ethylhexyl-P-Dimethyl-Aminobenzoate (Padimate-O)

CAS No.	21245-02-3
Source:	Sigma-Aldrich Company
Lot/Batch No.:	MKBF0590V
ILS Repository No.:	12-26
Formula:	$C_{17}H_{27}NO_2$

Description:	Colorless liquid
Purity:	98.3%
Dose Formulation:	Corn Oil
Storage:	
Test Substance:	Ambient temperature
Dose Formulation:	Ambient temperature protected from light
Stability:	
Dose Formulation:	Padimate-O in corn oil stored at ambient temperature was shown to be stable for 43 days (Blake, 2011c).

2.5 Reference Substance: 17α-Ethinyl Estradiol

CAS No.	57-63-6
Source:	Sigma Aldrich
Lot/Batch No.:	090M1241V
ILS Repository No.:	11-40
Formula:	$C_{20}H_{24}O_2$
Description:	White powder
Purity:	≥98%
Expiration Date:	February 2017
Dose Formulation:	ILS will prepare 17α-ethinyl estradiol in corn oil once at a dose level of 0.05 mg/mL and dispense into amber vials to be used daily during the study.
Storage:	
Reference Substance:	Ambient temperature and protected from light
Dose Formulation:	1-10°C protected from light

Stability:

Dose Formulation:	17α-Ethinyl estradiol in corn oil stored between 1-10°C was shown to be stable for 42 days (Messer, 2002).

2.6 Vehicle: Corn Oil

CAS No.:	8001-30-7
Source:	MP Biomedicals, LLC
Lot/Batch No.:	7862K
ILS Repository No.:	11-121
Formula:	$C_{27}H_{50}O_6$
Description:	Yellow oil
Storage:	Ambient temperature

2.7 Archival Samples

Approximately a 1 g sample of the test substance and approximately 1 mL of the vehicle and dose formulations will be stored between 0 and -30°C. Upon acceptance of the report by the Sponsor, archival dose formulation samples will be discarded.

2.8 Dose Formulation Analysis

Dose formulations will be prepared at ILS and analyzed at Midwest Research Institute (MRI) and Research Triangle Institute (RTI) International in accordance with GLP regulations as promulgated by the U.S. EPA GLP Regulations (40 CFR Part 160). Three samples of the test substance formulation (top, middle, and bottom) will be analyzed in duplicate for concentration and homogeneity. Concentration results will be acceptable if the mean concentration is within 10% of the target concentration. Homogeneity results will be acceptable if the coefficient of variation is ≤5%. Samples will be shipped to the following addresses for analysis prior to administration:

Ensulizole, Homosalate, and Padimate-O:
Research Triangle Institute International
[redacted]
Materials Handling Facility
East Institute Drive
Research Triangle Park, NC 27709

Avobenzone:
Midwest Research Institute
[redacted]
Program: NTP Chemistry Support
425 Volker Boulevard
Kansas City, MO 64110-2299

EXPERIMENTAL DESIGN

Eighty ovariectomized female Sprague-Dawley rats will be allocated to one of ten designated dose groups. The animals will be administered one of two dose levels of the four test substances, the vehicle control, or the reference substance (17α-ethinyl estradiol) for three consecutive days via oral gavage based upon daily body weights. Approximately 24-hours following the final dose administration, the animals will be humanely euthanized; the uterus excised, and wet and blotted uterine weights recorded. Changes in the uterine weights will be evaluated to determine if the test substance acts as an estrogen agonist.

3.1 Test System

Species:	Rat, *Rattus norvegicus*
Strain:	Sprague-Dawley Crl:CD®(SD) IGS
Source:	Charles River Laboratories International, Inc. (Raleigh, NC)
Number/Sex:	80 ovariectomized females, 6 weeks of age at ovariectomy. Surgical manipulation performed by Charles River Laboratories International, Inc.
Acclimation:	Animals will be allowed to recover from the surgical manipulation for 7 days at Charles River Laboratories International, Inc. prior to shipment to ILS. The animals will then be acclimated to ILS for at least 7 days in the room where the study will occur.
Estrous Cycle:	Vaginal smears will be collected for 5 consecutive days immediately preceding dose administration and evaluated for stage of the estrous cycle. If an animal indicates evidence of entering estrus, the animal will be evaluated to determine if it will be used on study.
Age at dose administration:	8-10 weeks of age

Weight at first day of dose administration:	175-275 grams
Identification:	Animals will be identified by the temporary numbers located on the animal's cage until allocation. Each animal will be uniquely identified by ear punch prior to dose administration.
Justification:	Animal model used is in accordance with OPPTS 890.1600: Uterotrophic Assay (U.S. EPA, 2009).

3.2 Animal Husbandry

All procedures are in compliance with the Animal Welfare Act Regulations, 9 CFR 1-4 and animals will be handled and treated according to the *Guide for the Care and Use of Laboratory Animals* (ILAR, 2011).

Housing (pre-allocation):	1 per cage
Housing (post-allocation):	2 per cage
Cage Type:	Polycarbonate with micro-isolator top
Cage Size:	23 cm wide by 44 cm long (1012 cm^2 area) and 21 cm high
Bedding:	Absorbent heat-treated hardwood bedding (Northeastern Bedding Corp., Warrensburg, NY)
Cage Changes:	At least once per week while single housed and twice per week while multi-housed.
Diet:	Teklad Global 16% Protein Rodent Diet (Teklad Diets, Madison WI) *ad libitum* Prior to shipment, rats are given Autoclaved Purina5L79 Rat and Mouse diet *ad libitum* at Charles River Laboratories International, Inc. A copy of the diet composition will be included in the raw data.

	Analysis:	The manufacturer's analytical results will be included in the raw data and reviewed prior to animal arrival to ensure the genistein equivalent content of genistein plus daidzein does not exceed 350 µg/g (as described by Owens et al., 2003).
	Archival:	A sample of the diet (~200 g) will be retained and stored between 0 and -30°C until acceptance of the final report and will then be discarded.
Water:		Reverse osmosis treated tap water (City of Durham, NC) *ad libitum*
	Supplied:	Glass water bottles with stainless steel sipper tubes
	Analysis:	The results of the current annual comprehensive chemical analyses of water from National Testing Laboratories, Inc. (Cleveland, OH) will be reviewed prior to initiation of the study and will be included in the raw data.
Water Bottle Changes:		At least once per week
Animal Room Conditions:		
	Temperature:	19-25°C
	Humidity	30-70%
	Lighting:	12/12 hour light/dark cycle
Animal Enrichment:		None

3.3 Allocation

The animals will be assigned to a dose group using a procedure that stratifies animals across groups by body weight such that mean body weight of each group is not statistically different from any other group using analysis of variance (ANOVA) (Statistical Analysis System version 9.2, SAS Institute, Cary, NC). Only clinically-healthy animals will be used for allocation.

3.4 Group Designations

Group Number	Animal Identification	Dose Group	Test Substance Dose Level (mg/kg/day)
1	01-08	Corn Oil Control	0
2	09-16	Homosalate	320
3	17-24	Homosalate	1000
4	25-32	Padimate-O	320
5	33-40	Padimate-O	1000
6	41-48	Avobenzone	320
7	49-56	Avobenzone	1000
8	57-64	Ensulizole	320
9	65-72	Ensulizole	1000
10	73-80	17α-Ethinyl estradiol	0.05

3.5 Dose Administration

The dose formulations will be administered via oral gavage at a dose volume of 5 mL/kg body weight for three consecutive days. Dose volume will be based on individual animal daily body weight.

The dose formulations will be administered on a staggered start for 3 consecutive days. The first four animals from each group will be dosed beginning on study day 1 through study day 3, and the second four animals from each group will begin on study day 2 of study through study day 4. Dose administration will occur 24-hours (± 2 hours) from the previous dose. The dosing sequence will be stratified across dose groups; one animal from each group and then repeated until all animals are dosed.

3.5.1 Justification of Route of Administration

Selection of the route of administration is in accordance with OPPTS 890.1600: Uterotrophic Assay (U.S. EPA, 2009). The relevant route of oral administration enables evaluation of potential estrogenic activity following metabolism of each of the test substances.

3.5.2 Justification of Dose Levels

Selection of the dose levels for each test substance was based on the available EC_{50} and OPPTS 890.1600 guidelines which state "to select doses that ensure animal survival and that are without significant toxicity or distress to the animals after three consecutive days of chemical administration up to a maximum dose of 1000 mg/kg/d".

3.5.3 Disposal of Dose Formulations

Dose formulations will be disposed of as hazardous material following dosing each day.

3.6 In-Life Animal Observations

Mortality/Moribundity:	Twice daily on weekdays, once daily on weekends/holidays
Clinical Observations:	Observed within 2 days of arrival, again for allocation of animals to study groups, daily prior to dose administration, and prior to euthanasia. A cage-side observation will occur 3 hours (± 30 minutes) after dose administration. If adverse clinical signs are seen additional observations may be recorded.
Body Weights:	Collected within 2 days of arrival, again for allocation of animals to study groups, daily prior to dose administration, and prior to euthanasia.

3.7 Termination

Moribunds/Unscheduled:	Tissue collection will not be performed on accidental deaths, moribund, or animals found dead during the acclimation period. Beginning on the first day of dose administration, any animals found moribund or dead will be necropsied under the supervision of a pathologist, facility veterinarian, or veterinary designee and cause of death will be determined and recorded, if possible. Moribund animals will be euthanized by carbon dioxide (CO_2) inhalation and death confirmed by cervical dislocation.
Scheduled:	Twenty-four hours (± 2 hours) after the final dose administration, animals will be humanely euthanized, in the same order as they

were dosed, by CO_2 asphyxiation with death confirmed by cervical dislocation. The first four animals from each group will euthanized on study day 4, and the second four animals from each group will be euthanized on study day 5.

Tissue Collection: The uterus will be removed and the ends of the uterine horns will be examined for the presence of any ovarian tissue. If ovarian tissue is observed it will be noted in the study records.

Gross observations of the uterus will be recorded.

Tissue Weights: The uterus will be excised, trimmed of excess adhering tissue and fat, weighed, and weights recorded to the nearest 0.0001 g. The uterus will then be pierced and blotted to remove the luminal contents, weighed (blotted), and weights recorded to the nearest 0.0001 g.

3.8 Statistical Analysis

Descriptive statistics (mean and standard deviation) will be calculated using MS Excel. Final body weight, body weight gain, and tissue weights will be analyzed using Statistical Analysis System version 9.2 (SAS Institute, Cary, NC). Studentized residual plots will be used to detect outliers and Levene's test will be used to assess homogeneity of variance. If the data is heterogeneous, then appropriate transformation will be performed and the data will be re-analyzed to assess homogeneity.

Final body weight, body weight gain, and uterine weights will be analyzed by ANOVA. Pair-wise comparisons will be made using a Dunnett's t test (one-tailed- uterine weights, two-tailed-final body weight and body weight gain). Statistically-significant effects will be reported when $p<0.05$.

Positive controls (17α-ethinyl estradiol) will be compared to controls by appropriate t tests.

3.9 Performance Criteria

Vehicle control blotted uterine weights should be less than 0.04% of body weight.

REPORT

The report will include all items in the study protocol as well as a comprehensive presentation of all data collected in the study.

RECORD RETENTION

All original data [including the original signed study protocol and all amendments (if any), test substance information, animal receipt records, animal caretaker records, observations, body weight records, clinical observations, etc.] and the original final report will be transferred to the National Toxicology Program Archives following finalization of the study report to the address below:

NTP Archives
[redacted]
615 Davis Drive, Suite 300
Durham, NC 27713

REFERENCES

Aillon, K. (2012). Dose Formulation Development Study of Avobenzone in Corn Oil. MRI Project Number-NTP ChemTask Number: 110730-Chem10987. Unpublished study report prepared by Midwest Research Institute.

Blake, J. (2011c). Padimate-O in Corn Oil Dose Formulation Development. RTI Project Number-ChemTask Number: 0212839.100.003.034-Chem11137. Unpublished study report prepared by Research Triangle Institute, International.

Blake, J. (2012a). Ensulizole in Corn Oil Dose Formulation Development. RTI Project Number-ChemTask Number: 0212839.200.003.066-Chem11145. Unpublished study report prepared by Research Triangle Institute, International.

Blake, J. (2012b). Homosalate in Corn Oil Dose Formulation Development. RTI Project Number-ChemTask Number: 0212839.200.003.063-Chem11139. Unpublished study report prepared by Research Triangle Institute, International.

Institute of Laboratory Animal Resources. (2011). *Guide for the Care and Use of Laboratory Animals.* National Academy Press, Washington, DC.

Messer, D. (2002). Dose Formulation Development Study for Ethinyl Estradiol in Corn Oil. Study Project Number-Task Number: 110100-197. Unpublished study report prepared by Midwest Research Institute.

Owens, W., Ashby, J., Odum, J., and Onyon, L. (2003). The OECD Program to Validate the Rat Uterotrophic Bioassay. Phase 2: Dietary Phytoestrogen Analyses. 111: 1559-1567.

U.S. EPA (Environmental Protection Agency).(2009). Endocrine Disruptor Screening Program Test Guidelines. OPPTS 890.1600: Uterotrophic Assay. EPA 740-C-09-0010. Office of Prevention, Pesticides and Toxic Substances, U.S. EPA., Washington, DC.

KEY PERSONNEL

Study Director:
Study Toxicologist:
Toxicology Study Manager:
Animal Facility Operations Manager:
Necropsy Manager:
Facility Veterinarian:
Health and Safety Manager:
Dose Formulations:

[redacted]

Appendix VIII Amendments, Deviations, and Notes to File

Integrated Laboratory Systems, Inc.

Protocol Amendment

ILS Project No.-Study No.: N135-247

Protocol Amendment No.: 1

Section Amended: 2.8

Amendment Made: Concentration results will be acceptable if the mean concentration is ≤ 15% of the target concentration, and homogeneity results will be acceptable if the coefficient of variation is ≤ 15% for Ensulizole.

Reason for Amendment: Ensulizole is a suspension in corn oil and the acceptable criteria for concentration and homogeneity parameters are modified to account for the properties of the chemical.

[redacted]

Chief, Toxicology Branch
National Toxicology Program, NIEHS

4/2/12
Date

[redacted]

Contract Office Technical Representative
National Toxicology Program, NIEHS

4/2/12
Date

[redacted]

Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

6/2/12
Date

[redacted]

Study Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

4-2-12
Date

Page 1 of 1

Integrated Laboratory Systems, Inc.

Protocol Deviation 1

ILS Project No.-Study No.:	N135-247
Protocol Section Deviated from:	3.2
Nature of Deviation:	Relative humidity was out of range on the following dates: 27 March 2012
Reason for Deviation:	Slight fluctuations in the HVAC system.
Corrective Action:	The HVAC system was evaluated to ensure proper functioning.
Impact on Study:	There is no significant impact on the study since the slightly lower humidity did not cause any abnormal clinical observations in the animals.
Protocol Section Deviated from:	3.2
Nature of Deviation:	Temperature was out of range on : 04 April 2012
Reason for Deviation:	Slight fluctuations in the HVAC system.
Corrective Action:	The HVAC system was evaluated to ensure proper functioning.
Impact on Study:	There is no significant impact on the study since the slightly higher temperature did not cause any abnormal clinical observations in the animals.
Protocol Section Deviated from:	2.5
Nature of Deviation:	Concentration of 17α-ethinyl estradiol was listed in protocol as 0.05 mg/mL.
Reason for Deviation:	Typographical
Corrective Action:	None.

Page 1 of 2

Impact on Study:

There is no significant impact on the study since 17α-ethinyl estradiol was made at the correct concentration of 0.01 mg/mL.

[redacted]

4-18-12

Date

Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

Integrated Laboratory Systems, Inc.

SOP Deviation 1

ILS Project No.-Study No.:	N135-247
SOP No.-Mod. No. Deviated:	718-13

SOP Section Deviated:	II.A.1
Nature of Deviation:	Research Assistant did not notify supervisor/study director of an out of range temperature on 04 April 2012.
Reason for Deviation:	Research Assistant oversight.
Corrective Action:	Staff was verbally reminded to notify supervisor/study director when out of range temperatures occur.
Impact on Study:	None, the slightly out of range temperature did not cause any abnormal clinical observations in any of the study animals.

[redacted]

Study Director, ILS, Inc.

4/19/12

Date

ILS-A-064
Last Revised: 08/08/12

Integrated Laboratory Systems, Inc.
Note to File

ILS Project No.-Study No.: N135-247

Note to File No.: 1

The preliminary formulations concentrations received for Avobenzone 20 mg/ml (64 mg/ml) showed the results of the formulation to be ~32 mg/ml, and therefore ILS asked MRI to reanalyzed the sample. When the sample was reanalyzed, it was determined to be double what was originally determined (as detailed in the dose formulation report, Appendix II). The reanalyzed sample concentration and homogeneity results were reported in the final study report.

[redacted]

8-8-12
Date

Study Director
Integrated Laboratory Systems, Inc.

Page **1** of **1**

Appendix IX
Baseline Positive Control Test Data

Group No.	Animal No.	Treatment	Abs. Wet Uterine Weight (mg)	Abs. Blotted Uterine Weight (mg)	Rel. Wet Uterine Weight	Rel. Blotted Uterine Weight
1	01	Reference Item	23.4	19.2	0.0434	0.0356
1	02		26.6	23.4	0.0464	0.0408
1	03		26.1	21.3	0.0455	0.0371
1	04		36.5	30.8	0.0607	0.0512
1	05		19.5	15.4	0.0319	0.0252
1	06		38.7	32.0	0.0631	0.0522
1	07		35.6	28.9	0.0590	0.0479
1	08		30.3	27.0	0.0474	0.0423
		Mean	**29.6**	**24.8**	**0.0497**	**0.0415**
		St. dev.	**6.9**	**5.9**	**0.0106**	**0.0090**
2	09	17α-Ethinyl estradiol (0.1 µg/kg-day)	30.8	26.7	0.0606	0.0526
2	10		29.5	25.6	0.0526	0.0456
2	11		35.4	30.3	0.0638	0.0546
2	12		27.7	20.3	0.0471	0.0345
2	13		36.7	31.9	0.0614	0.0533
2	14		44.2	35.5	0.0674	0.0541
2	15		37.1	33.2	0.0603	0.0540
2	16		36.5	31.7	0.0545	0.0473
		Mean	**34.7**	**29.4**	**0.0585**	**0.0495**
		St. dev.	**5.3**	**4.9**	**0.0066**	**0.0069**
3	17	17α-Ethinyl estradiol (0.3 µg/kg-day)	71.8	62.9	0.1287	0.1127
3	18		65.2	58.8	0.1128	0.1017
3	19		78.8	68.7	0.1342	0.1170
3	20		78.3	69.8	0.1345	0.1199
3	21		71.5	62.6	0.1124	0.0984
3	22		71.6	64.0	0.1170	0.1046
3	23		77.2	67.6	0.1156	0.1012
3	24		93.2	83.5	0.1421	0.1273
		Mean	**76.0**	**67.2**	**0.1247**	**0.1104**
		St. dev.	**8.3**	**7.5**	**0.0116**	**0.0104**
4	25	17α-Ethinyl estradiol (1.0 µg/kg-day)	161.2	112.2	0.3224	0.2244
4	26		140.0	100.4	0.2607	0.1870
4	27		198.6	126.6	0.3611	0.2302
4	28		112.8	83.2	0.1912	0.1410
4	29		219.2	126.4	0.3741	0.2157
4	30		191.0	107.4	0.3111	0.1749
4	31		216.5	127.2	0.3270	0.1921
4	32		169.0	112.6	0.2649	0.1765
		Mean	**176.0**	**112.0**	**0.3016**	**0.1927**
		St. dev.	**37.4**	**15.3**	**0.0600**	**0.0298**
5	33	17α-Ethinyl estradiol (3.0 µg/kg-day)	174.5	115.3	0.3035	0.2005
5	34		282.1	140.0	0.5456	0.2708
5	35		204.6	111.0	0.3583	0.1944
5	36		135.8	99.7	0.2434	0.1787
5	37		327.4	133.7	0.5568	0.2274
5	38		288.5	134.1	0.4522	0.2102
5	39		142.3	83.6	0.2333	0.1370
5	40		175.4	116.7	0.2866	0.1907
		Mean	**216.3**	**116.8**	**0.3725**	**0.2012**
		St. dev.	**73.1**	**19.1**	**0.1302**	**0.0385**