FINAL REPORT

Study Title

The Hershberger Bioassay for Ensulizole and Avobenzone

ILS Project-Study Number

N135-248

Guideline Reference Number

OPPTS 890.1400

Author

[redacted]

Performing Laboratory

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Sponsor

National Institute of Environmental Health Sciences
P.O. Box 12233
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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:

Flutamide and testosterone propionate were purchased commercially and 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 flutamide and/or testosterone propionate 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 MRI Global (Kansas City, MO) and analysis for Ensulizole 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/Submitter: [redacted]

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

Typed Name of Company: National Institute of Environmental Health Sciences

This final report has been reviewed by:

[redacted] 11-7-12

Date:

Study Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

QUALITY ASSURANCE INSPECTION STATEMENT

Laboratory Project ID - Study No.: N135-248

Study Title: The Hershberger Bioassay for Ensulizole and Avobenzone

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:	21 March 2012	21 March 2012/21 March 2012
Dose Administration:	06 April 2012	06 April 2012/09 April 2012
Data Audit:	01-05 June 2012	05 June 2012/07 June 2012
Draft Report:	11-12 June 2012	13 June 2012/20 June 2012
Final Report:	05 November 2012	05 November 2012/05 November 2012

[redacted] 11/07/2012

Quality Assurance Auditor Date

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SUMMARY

The purpose of this Hershberger Bioassay was to screen Ensulizole and Avobenzone for its ability to elicit biological activities consistent with androgen agonists, antagonists, or 5α-reductase inhibitors using the adult castrated rat model. This assay is one of a suite of assays selected for Tier 1 Screening as part of U.S. EPA's Endocrine Disruptor Screening Program (EDSP).

One hundred four castrated male Sprague-Dawley (SD) rats were allocated to 1 of 13 designated dose groups. To evaluate the test substance for agonist properties, animals were administered 1 of 2 dose levels (320 or 1000 mg/kg/day) of Ensulizole or Avobenzone, the vehicle control (corn oil), or the agonist reference substance testosterone propionate (TP, 0.4 mg/kg/day). To evaluate Ensulizole and Avobenzone for antagonist properties animals were co-administered 1 of 3 dose levels (100, 320, or 1000 mg/kg/day) of Ensulizole or Avobenzone with TP (0.4 mg/kg/day). Flutamide (FT, 3 mg/kg/day) with TP (0.4 mg/kg/day) was utilized as the antagonist positive control.

Animals were dosed for 10 consecutive days via oral gavage (Ensulizole, Avobenzone, or FT) and subcutaneous injection (TP). Approximately 24-hours following the final dose administration, the animals were humanely euthanized; the glans penis, ventral prostate, levator ani plus bulbocavernous muscle complex (LABC), Cowper's gland, and seminal vesicle with coagulating gland with fluid were excised and weighed. Changes in androgen-dependent tissue weights were evaluated to determine the ability of Ensulizole or Avobenzone to act as androgen agonists/antagonists, or inhibitors of 5α-reductase.

In the agonist assay, final body weight, body weight gain, glans penis, ventral prostate, LABC, Cowper's gland, or seminal vesicle with coagulating gland with fluid were not significantly different in animals administered 320 or 1000 mg/kg/day Ensulizole compared to control animals (corn oil). In the antagonist assay, final body weight, body weight gain, glans penis, ventral prostate, LABC, Cowper's gland, or seminal vesicle with coagulating gland with fluid were not significantly different in animals co-administered 100, 320, or 1000 mg/kg/day Ensulizole and TP compared to vehicle control animals (corn oil and TP).

In the agonist assay, final body weight, body weight gain, glans penis, ventral prostate, LABC, Cowper's gland, or seminal vesicle with coagulating gland with fluid were not significantly different in animals administered 320 or 1000 mg/kg/day Avobenzone compared to control animals (corn oil). In the antagonist assay, final body weight, body weight gain, glans penis, ventral prostate, LABC, Cowper's gland, or seminal vesicle with coagulating gland with fluid were not significantly different in animals co-administered 100, 320, or 1000 mg/kg/day Avobenzone and TP compared to vehicle control animals (corn oil and TP).

Based on these findings using the castrated rat model, oral administration of Ensulizole or Avobenzone, up to the limit dose of 1000 mg/kg/day, did not show any androgen agonist/antagonist activity, or 5α-reductase inhibition.

INTRODUCTION

1.1 Study Title

The Hershberger Bioassay for Ensulizole and Avobenzone

1.2 Laboratory Project Identification

ILS Project No.-Study No.: N135-248

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). 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 activity will be made on a weight-of-evidence basis taking into account data from the Tier 1 assays and other available scientifically-relevant information. 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 Hershberger Bioassay (OPPTS 890.1400) is used as an *in vivo* screening assay for androgen agonists, androgen antagonists, and 5α-reductase inhibitors and is one of four *in vivo* mammalian assays in the EDSP Tier 1 battery of assays.

1.4 Purpose of the Study

The purpose of the Hershberger Bioassay assay was to screen Ensulizole and Avobenzone for their ability to elicit biological activities consistent with androgen agonists, antagonists, or 5α-reductase inhibitors using the castrated rat model (OPPTS 890.1400).

1.5 Sponsor

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

NIEHS Investigator
[redacted]

Telephone No.: [redacted]
Email: [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 USA

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

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: 29 March 2012
Experimental Start Date: 06 April 2012
Experimental End Date: 17 April 2012

TEST SUBSTANCE

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

CAS No. 27503-81-7

Source: Sigma-Aldrich Company

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 formulations were prepared at ILS two times. Ensulizole formulations were prepared using corn oil as the vehicle at concentrations of 20, 64, or 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 formulations were prepared at ILS one time. Avobenzone formulations were prepared using corn oil as the vehicle at concentrations of 20, 64, or 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:	Avobenzone in corn oil stored at ambient temperature was shown to be stable for 42 days (Aillon, 2012a).

2.3 Reference Substance: Testosterone Propionate (TP)

CAS No.	57-85-2
Source:	Sigma-Aldrich Company
Lot/Batch No.:	051M1803V
Expiration:	06 March 2014
ILS Repository No.:	09-26
Formula:	$C_{22}H_{32}O_3$
Description:	White to off-white powder
Purity:	100%
Dose Formulation:	TP was prepared at ILS in corn oil once at a dose level of 0.8 mg/mL and dispensed into vials used daily during the study.

Storage:

Reference Substance:	Ambient temperature, protected from light
Dose Formulation:	Between 1-10°C

Stability:

Dose Formulation:	TP in corn oil held between 1-10°C was shown to be stable for 14 days (Smith, 2011).

2.4	**Reference Substance:**	**Flutamide (FT)**
	CAS No.	13311-84-7
	Source:	Sigma-Aldrich Company
	Lot/Batch No.:	021M1406V
	Expiration:	27 June 2012
	ILS Repository No:	11-77
	Formula:	$C_{11}H_{11}F_3N_2O_3$
	Description:	Yellow powder
	Purity:	99.5%
	Dose Formulation:	FT was prepared at ILS in corn oil once at a dose level of 0.6 mg/mL and dispensed into vials used daily during the study.
	Storage:	
	Reference Substance:	Ambient temperature, protected from light
	Dose Formulation:	Between 1-10°C
	Stability:	
	Dose Formulation:	FT in corn oil stored between 1-10°C was shown to be stable for 42 days (Graves, 2001).
2.5	**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

2.6 Archival Samples

Approximately a 1 g sample of the neat test substances and 1 mg of the reference substances are stored between 0 and -30°C. One mL of the vehicle and each dose formulation are stored between 0 and -30°C until acceptance of the final report; after acceptance of the report by the Sponsor archival dose formulations will be discarded. The archival test substance and reference substance samples will be maintained by ILS for 5 years following submission of the final report to the Sponsor.

2.7 Dose Formulation Analysis

Dose formulations were prepared at ILS and sent and analyzed at Research Triangle Institute (RTI) International and Midwest Research Institute (MRI) in accordance with GLP regulations as promulgated by the U.S. EPA (40 CFR Part 160).

Ensulizole:
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

Three samples (top, middle, and bottom) of the test substance were analyzed for concentration and homogeneity. Concentration results were acceptable if the mean concentration was within 10% of the target concentration. Homogeneity results were acceptable if the coefficient of variation was less than ≤15%.

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:	104/castrated males; surgical manipulation was performed by Charles River Laboratories International, Inc. Rats were postnatal day (PND) 45 at surgery. Note: PND 0 is the day of birth
Date of birth:	07 February 2012
Age at arrival:	PND 51
Acclimation:	Animals were acclimated in the study room for 7 days.
Age at dose administration:	PND 59/60
Weight at dose administration:	249.7 – 330.6 g (See Protocol Deviation 1)
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 was in accordance with OPPTS 890.1400: Hershberger Bioassay (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 Purina 5L79 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 of feed.
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:	21.4-23.5°C
Humidity	28-66% (See Protocol Deviation 1)
Lighting:	12/12 hour light/dark cycle

Cleaning: The room was sanitized within 1 day of animal receipt.

Enrichment: None

STUDY DESIGN

4.1 Allocation

The animals were assigned to a dose group using a procedure that allocated 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].

4.2 Group Designation

Table 1. Androgen Agonist - Group Number, Animal Identification, Dose Group and Level

Group Number	Animal Identification	Test Substance / Control	Test Substance Dose Level (mg/kg/day)
1	001-008	Vehicle Control (Corn Oil)	0
2	009-016	Ensulizole	320
3	017-024	Ensulizole	1000
4	025-032	Avobenzone	320
5	033-040	Avobenzone	1000

Table 2. Androgen Antagonist - Group Number, Animal Identification, Dose Group and Level

Group Number	Animal Identification	Test/Reference Substance / Control	Test/Reference Substance Dose Level (mg/kg/day)
6[§]	041-048	Vehicle Control (Corn Oil) + TP	0 + 0.4
7	049-056	Ensulizole + TP	100 + 0.4
8	057-064	Ensulizole + TP	320 + 0.4
9	065-072	Ensulizole + TP	1000 + 0.4
10	073-080	Avobenzone + TP	100 + 0.4
11	081-088	Avobenzone + TP	320 + 0.4
12	089-096	Avobenzone + TP	1000 + 0.4
13	097-104	FT + TP	3 + 0.4

[§] Group served as the positive control for the agonist assay and control in the antagonist assay

4.3 Dose Administration

The test substances, FT, or corn oil (vehicle control) dose formulations were administered by oral gavage at a dose volume of 5 mL/kg body weight. TP dose formulations were administered by subcutaneous injection into the dorsoscapular region at a dose volume of 0.5 mL/kg body weight. In co-administered animals, oral gavage preceded subcutaneous injections.

The dose formulations were administered on a staggered start for 10 consecutive days (PND 59/60 through PND 68/69). The first 4 animals from each group were dosed beginning on PND 59 and the second 4 from each group on PND 60. Dosing occurred 24-hours (± 2 hours) from the previous dose. Dose volume was determined on individual animal daily body weight. The dosing sequence was stratified across dose groups; 1 animal from each group and then repeated until all animals were dosed.

4.3.1 Justification of Route of Administration

Selection of the route of administration is in accordance with OPPTS 890.1400: Hershberger Bioassay (U.S. EPA, 2009).

4.3.2 Justification of Dose Levels

> OPPTS 890.1400 specifies to select doses that ensure animal survival and that are without significant toxicity or distress to the animals after 10 consecutive days of chemical administration, and the highest dose should not cause a reduction in the final body weight of the animals greater than 10% of control weight.

Selection of the highest dose level for each test substance was based upon the available LD_{50} and/or acute toxicity information in order to avoid death, severe suffering, or distress in the animals and second, takes into consideration available information on the doses used in other studies. In general, the highest dose should not cause a reduction in the final body weight of the animals greater than 10% of control body weight. The highest dose should ensure animal survival and that is without significant toxicity or distress to the animals after 10 consecutive days of administration up to a maximal dose of 1000 mg/kg/day.

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.
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.
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: Gross observations of the tissues that were excised for tissue weights were recorded.

Tissue Weights: The following tissues were excised, trimmed of excess adhering tissue and fat, and weighed to the nearest 0.0001 g.

1. Ventral Prostate
2. Seminal vesicle with coagulating gland with fluid
3. Levator ani plus bulbocavernous muscle complex (LABC)
4. Cowper's gland (weighed as a pair)
5. Glans penis

4.6 Statistical Analysis

Descriptive statistics (mean, standard deviation, and coefficient of variance) were calculated using MS Excel. Final body weight, body weight gain, and tissue weights were analyzed using SAS version 9.2 (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 androgen-dependent tissue weights were analyzed by one-way ANOVA followed by pair-wise comparisons using a Dunnett's one tailed t test (tissues weights) or Dunnett's two tailed t test (final body weight and body weight gain). Positive controls were analyzed by 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

Actual concentration and homogeneity results of each dose formulation used in the study were within the acceptance criteria (Appendix II).

Table 3. Analytical Results for Dose Formulations
Preparation Date: 22 March 2012

Test Substance	Nominal Dose Formulation Concentration (mg/mL)	Actual Dose Formulation Concentration* (mg/mL) [Percent from Nominal]	Percent CV* (Homogeneity)	Nominal Dose Level (mg/kg/day)	Actual Dose Level (mg/kg/day)
Ensulizole	200	189.0 [5.5]	10.3	1000	945.0
Avobenzone	20	20.7 [3.5]	2.2	100	103.5
Avobenzone	64	63.3 [1.1]	1.0	320	316.5
Avobenzone	200	209.5 [4.8]	0.8	1000	1047.5

*Source: Allion (2012b); Blake (2012b)
Abbreviation: CV – coefficient of variation

Table 4. Analytical Results for Dose Formulations
Preparation Date: 03 April 2012

Test Substance	Nominal Dose Formulation Concentration (mg/mL)	Actual Dose Formulation Concentration* (mg/mL) [Percent from Nominal]	Percent CV* (Homogeneity)	Nominal Dose Level (mg/kg/day)	Actual Dose Level (mg/kg/day)
Ensulizole	20	18.5 [7.5]	11.2	100	92.5
Ensulizole	64	63.9 [0.2]	8.6	320	319.5

*Source: Blake (2012b)
Abbreviation: CV – coefficient of variation

5.2 In Life Animal Observations

Mortality/Moribundity

Androgen Agonist (Groups 1-6[§])

All animals survived to scheduled euthanasia with none showing signs of moribundity.

Androgen Antagonist (Groups 6[§]-13)

All animals survived to the scheduled euthanasia with none showing signs of moribundity.

Clinical Observations

Clinical observations were recorded for all animals 24-hours post-dose administration. No adverse observations were noted throughout the study. Individual animal data are listed in Appendix IV.

Androgen Agonist (Groups 1-6[§])

No clinical signs of toxicity were observed in any animals administered vehicle control (corn control), 320, or 1000 mg/kg/day Ensulizole or Avobenzone (Groups 1-5), and TP alone (Group 6) 24-hours post-dose.

[§] Group 6 served as the positive control for the agonist assay and control in the antagonist assay

Androgen Antagonist (Groups 6[§]-13)

No clinical signs of toxicity were observed in any animals administered vehicle control (corn oil) and TP (Group 6) or co-administered 100, 320, or 1000 mg/kg/day Ensulizole or Avobenzone and TP (Groups 7-12) or FT and TP (Group 13) 24-hours post-dose.

Body Weights

Group mean initial and final body weights and body weight changes for animals euthanized following 10 consecutive days of Ensulizole or Avobenzone administration are presented in Table 5 (agonist assay) and Table 6 (antagonist assay). Individual animal data are listed in Appendix V.

Androgen Agonist (Groups 1-6[§], Table 5)

No significant change in mean final body weight or body weight gain was observed in animals administered 320 or 1000 mg/kg/day Ensulizole or Avobenzone (Groups 2-5) compared to vehicle control group (Group 1). Positive control animals administered vehicle control and TP (Group 6) showed a statistically significant increase in final mean body weight and body weight gain as compared to the vehicle control group (Group 1).

Androgen Antagonist (Groups 6[§]-13, Table 6)

No significant change in body weight or body weight gain was observed in animals co-administered 100, 320, or 1000 mg/kg/day Ensulizole or Avobenzone and TP (Groups 7-12) compared to vehicle control and TP (Group 6). Positive control animals administered FT and TP (Group 13) had a significantly lower body weight gain than control animals (Group 6).

[§] Group 6 served as the positive control for the agonist assay and control in the antagonist assay

Table 5. Androgen Agonist; Body Weight Changes

Dose Group	Test/Reference Substance / Control	Test Substance Dose Level (mg/kg/day)	n	Initial Body Weight Mean (g) ± SD	Final Body Weight Mean (g) ± SD	Body Weight Gain Mean (g) ± SD	Final Body Weight (% of Control)
1	Vehicle Control (Corn Oil)	0	8	285.9 ± 20.3	325.0 ± 26.1	39.1 ± 7.8	-
2	Ensulizole	320	8	287.3 ± 21.4	342.8 ± 26.1	55.5 ± 30.9	105.5
3	Ensulizole	1000	8	287.8 ± 20.5	322.4 ± 30.9	34.6 ± 11.5	99.2
4	Avobenzone	320	8	285.6 ± 18.3	317.7 ± 20.0	32.1 ± 10.1	97.8
5	Avobenzone	1000	8	287.7 ± 19.4	319.6 ± 27.1	31.9 ± 9.7	98.3
6[§]	Vehicle Control + TP (Positive Control)	0 + 0.4	8	286.1 ± 16.3	**352.7 ± 19.5**[†]	**66.6 ± 10.0**[†]	-

Abbreviation: SD - standard deviation; TP- testosterone propionate
[†]Statistically significant ($p<0.05$) compared to the vehicle control mean (t test)
[§]Group served as the positive control for the agonist assay and control in the antagonist assay

Table 6. Androgen Antagonist; Body Weight Changes

Dose Group	Test/Reference Substance / Control	Test Substance Dose Level (mg/kg/day)	n	Initial Body Weight Mean (g) ± SD	Final Body Weight Mean (g) ± SD	Body Weight Gain Mean (g) ± SD	Final Body Weight (% of Control)
6[§]	Vehicle Control + TP (Control)	0 + 0.4	8	286.1 ± 16.3	352.7 ± 19.5	66.6 ± 10.0	-
7	Ensulizole + TP	100 + 0.4	8	287.9 ± 16.0	355.7 ± 18.5	67.8 ± 10.6	100.9
8	Ensulizole + TP	320 + 0.4	8	285.7 ± 16.6	364.5 ± 21.2	78.8 ± 13.7	103.3
9	Ensulizole + TP	1000 + 0.4	8	286.2 ± 14.1	358.9 ± 21.4	70.3 ± 18.3	101.8
10	Avobenzone + TP	100 + 0.4	8	288.1 ± 15.9	360.3 ± 24.3	72.2 ± 9.7	102.1
11	Avobenzone + TP	320 + 0.4	8	286.2 ± 15.5	352.8 ± 22.4	66.6 ± 7.6	100.0
12	Avobenzone + TP	1000 + 0.4	8	286.9 ± 17.2	341.7 ± 26.9	54.8 ± 16.9	96.9
13	FT + TP (Positive Control)	3 + 0.4	8	285.1 ± 14.5	**344.6 ± 23.0**[†]	**59.5 ± 12.1**[†]	-

Abbreviation: SD - standard deviation; TP- testosterone propionate; FT- flutamide
[†]Statistically significant ($p<0.05$) compared to the vehicle control mean (t test)
[§]Group served as the positive control for the agonist assay and control in the antagonist assay

5.3 Necropsy

Gross Observations

One animal in Group 3 was observed with bladder mucosa thickened and containing multiple calculi. All remaining animals were observed as normal.

Tissue Weights

Group mean weights of glans penis, Cowper's gland, LABC, ventral prostate and seminal vesicle for animals euthanized following 10 consecutive days of

Ensulizole or Avobenzone administration are presented in Table 7 (agonist assay) and Table 8 (antagonist assay). Individual animal tissue weight data are listed in Appendix VI.

Androgen Agonist (Groups 1-6[§], Table 7)

Administration of 320 or 1000 mg/kg/day Ensulizole or Avobenzone (Groups 2-5) did not affect glans penis, Cowper's gland, LABC, ventral prostate, or seminal vesicle weights in the agonist assay. All five androgen-dependent tissues weights were significantly increased in the positive control group (vehicle and TP; Group 6) as compared to vehicle control group (Group 1).

Androgen Antagonist (Groups 6[§]-13, Table 8)

Co-administration of 100, 320, or 1000 mg/kg/day Ensulizole or Avobenzone and TP (Groups 7-12) did not affect glans penis, Cowper's gland, LABC, ventral prostate, or seminal vesicle weights compared to vehicle control and TP (Group 6) in the antagonist assay. All five androgen-dependent tissues weights were significantly decreased in the positive control group, (FT and TP; Group 13) compared to the vehicle control and TP control group (Group 6).

[§]Group 6 served as the positive control for the agonist assay and control in the antagonist assay

Table 7. Androgen Agonist; Androgen Dependent Tissue Weights

Dose Group	Test/Reference Substance / Control	Test Substance Dose Level (mg/kg/day)	n	Glans Penis Weight (mg) Mean ± SD (CV)	Cowper's Gland Weight (mg) Mean ± SD (CV)	LABC Weight (mg) Mean ± SD (CV)	Ventral Prostate Weight (mg) Mean ± SD (CV)	Seminal Vesicle Weight (mg) Mean ± SD (CV)
1	Vehicle Control	0	8	59.2 ± 5.7 (9.7)	7.8 ± 2.9 (37.0)	133.9 ± 4.1 (29.2)	25.3 ± 6.6 (26.1)	62.4 ± 4.5 (7.2)
2	Ensulizole	320	8	61.0 ± 4.5 (7.4)	7.4 ± 2.7 (36.5)	145.3 ± 13.5 (9.3)	19.1 ± 4.0 (20.9)	60.2 ± 10.9 (18.1)
3	Ensulizole	1000	8	63.3 ± 6.7 (10.6)	7.5 ± 1.7 (22.5)	140.6 ± 23.6 (16.8)	21.6 ± 6.2 (28.7)	66.6 ± 10.6 (15.9)
4	Avobenzone	320	8	60.7 ± 4.3 (7.1)	7.8 ± 2.9 (36.9)	149.1 ± 45.3 (30.4)	19.3 ± 2.6 (13.6)	58.3 ± 12.7 (21.8)
5	Avobenzone	1000	8	59.9 ± 4.9 (8.2)	6.3 ± 1.4 (22.9)	139.3 ± 26.8 (19.2)	20.6 ± 5.0 (24.4)	60.3 ± 7.1 (11.8)
6[§]	Vehicle Control + TP (Positive Control)	0 + 0.4	8	**99.8 ± 7.3[†] (7.3)**	**44.8 ± 13.2[†] (29.4)**	**440.5 ± 63.4[†] (14.4)**	**176.4 ± 30.6[†] (17.3)**	**765.1 ± 144.7[†] (18.9)**

Abbreviations: SD - standard deviation; LABC-levator ani plus bulbocavernous muscle complex; CV- Coefficient of Variation; TP- testosterone propionate

[†]Statistically significant ($p<0.05$) compared to the vehicle control mean (t test)

[§]Group served as the positive control for the agonist assay and control in the antagonist assay

Table 8 Androgen Antagonist; Androgen Dependent Tissue Weights

Dose Group	Test/Reference Substance / Control	Test Substance Dose Level (mg/kg/day)	n	Glans Penis Weight (mg) Mean ± SD (CV)	Cowper's Gland Weight (mg) Mean ± SD (CV)	LABC Weight (mg) Mean ± SD (CV)	Ventral Prostate Weight (mg) Mean ± SD (CV)	Seminal Vesicle Weight (mg) Mean ± SD (CV)
6[§]	Vehicle Control + TP (Control)	0 + 0.4	8	99.8 ± 7.3 (7.3)	44.8 ± 13.2 (29.4)	440.5 ± 63.4 (14.4)	176.4 ± 30.6 (17.3)	765.1 ± 144.7 (18.9)
7	Ensulizole + TP	100 + 0.4	8	99.4 ± 5.5 (5.5)	47.0 ± 9.8 (20.8)	423.7 ± 53.0 (12.5)	228.9 ± 33.5 (14.7)	805.1 ± 123.3 (15.3)
8	Ensulizole + TP	320 + 0.4	8	99.2 ± 4.9 (5.0)	54.0 ± 9.2 (17.0)	444.1 ± 49.9 (11.2)	216.8 ± 25.6 (11.8)	859.5 ± 98.0 (11.4)
9	Ensulizole + TP	1000 + 0.4	8	102.9 ± 7.4 (7.2)	50.8 ± 7.8 (15.3)	464.5 ± 65.5 (14.1)	246.6 ± 72.5 (29.4)	88.4 ± 98.3 (11.1)
10	Avobenzone + TP	100 + 0.4	8	102.0 ± 4.3 (4.3)	50.9 ± 7.4 (14.6)	403.1 ± 52.6 (13.0)	204.0 ± 24.8 (12.1)	826.7 ± 129.9 (15.7)
11	Avobenzone + TP	320 + 0.4	8	97.1 ± 6.7 (6.9)	49.5 ± 8.1 (16.4)	397.4 ± 33.1 (8.3)	216.6 ± 19.1 (8.8)	756.7 ± 111.4 (14.7)
12	Avobenzone + TP	1000 + 0.4	8	100.3 ± 4.4 (4.4)	50.4 ± 10.6 (21.1)	422.9 ± 40.3 (9.5)	201.3 ± 15.5 (7.7)	770.4 ± 92.5 (12.0)
13	FT + TP (Positive Control)	3 + 0.4	8	**72.5 ± 9.3[†] (12.8)**	**19.9 ± 5.3[†] (26.7)**	**195.8 ±24.6[†] (12.6)**	**50.4 ± 7.9[†] (15.6)**	**143.3 ± 65.9[†] (46.0)**

Abbreviations: SD - standard deviation; LABC-levator ani plus bulbocavernous muscle complex; CV- Coefficient of Variation; TP- testosterone propionate; FT-flutamide

[†]Statistically significant ($p<0.05$) compared to the control mean (t test)

[§]Group served as the positive control for the agonist assay and control in the antagonist assay

Performance Criteria

Agonist

All tissue CVs met performance criteria for the antagonist assay (Tables 7 and 9).

Antagonist

All tissue CVs met performance criteria for the antagonist assay (Tables 8 and 9).

Table 9. Maximum Allowable Coefficient of Variations

Tissue	Androgen Agonist	Androgen Antagonist
Glans Penis	22%	17%
Cowper's Glands	55%	35%
LABC	30%	20%
Ventral Prostate	45%	40%
Seminal Vesicle	40%	40%

Source: U.S. EPA (2009)

CONCLUSION

Castrated SD male rats were orally administered Ensulizole or Avobenzone alone or co-administered subcutaneously with TP for 10 consecutive days. Approximately 24-hours following the final dose administration, the animals were humanely euthanized; the glans penis, ventral prostate, LABC, Cowper's gland, and seminal vesicle with coagulating gland with fluid were excised and weighed.

In the agonist assay, mean final body weight and body weight gain were not significantly different in animals administered 320 or 1000 mg/kg/day Ensulizole or Avobenzone compared to vehicle controls. Dose levels of 320 or 1000 mg/kg/day Ensulizole or Avobenzone did not increase androgen-dependent tissue weights compared to the weights of these tissues in the vehicle control animals.

In the antagonist assay, final body weights and body weight gain of animals co-administered 100, 320, or 1000 mg/kg/day Ensulizole or Avobenzone and TP were not statistically different as compared to the vehicle control animals (corn oil and TP). Ensulizole or Avobenzone, administered at dose levels of 100, 320, or 1000 mg/kg/day, co-administered with TP at did not decrease androgen-dependent tissue weights.

Based on these findings oral administration of Ensulizole or Avobenzone, up to the limit dose of 1000 mg/kg/day, did not show any androgen agonist/ antagonist activity, or 5α-reductase inhibition properties.

REFERENCES

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KEY PERSONNEL

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

Appendix I:

Certificate of Analysis

N135-248
[redacted] 3-22-12

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]/Solon/MPBIO 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

Free Fatty Acid: 0.045%
Peroxide: 0.5 meq/kg
Iodine: 126.85
Cold Test: 5.5 Clear & Brilliant
Additives: None

[redacted]

08/17/2010

[redacted]

MP Biomedicals, LLC.
Technical Director

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mailto:biotech@mpbio.com
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Online Ordering, MSDSs, certificates of analysis and data sheets now available on our web site
Technical Service: 1-800-279-5490 (440-337-1200) Customer Service: 1-800-854-0530 (440-337-1200)

UNIVERSAL PRESERV A-CHEM INC.

N135-248

[redacted] 3-22-12

Chemicals for Food, Pharmaceutical, Cosmetic and Industrial Trades

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: [redacted]

Approved By: [redacted] Technical Director

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

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NTP Analytical Chemistry Services

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Telephone 919.541.6730 or 919.541.5975 • Fax 919.485.2650 • www.rti.org

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

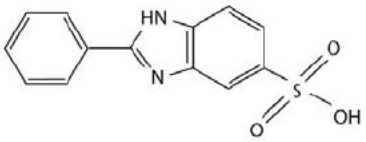
Submitted to:

[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] (RTI)
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.

Certificate of Analysis

SIGMA-ALDRICH

Product Name	2-Phenyl-5-benzimidazolesulfonic acid, 96%
Product Number	437166
Product Brand	ALDRICH
CAS Number	27503-81-7
Molecular Formula	$C_{13}H_{10}N_2O_3S$
Molecular Weight	274.30

TEST	SPECIFICATION	LOT 05117JE RESULTS
APPEARANCE	WHITE TO OFF-WHITE POWDER, CRYSTALS.	WHITE POWDER
INFRARED SPECTRUM		CONFORMS TO STRUCTURE.
TITRATION	95.5%-104.5% (WITH NAOH)	100.2% (WITH NAOH)
HIGH PRESSURE LIQUID CHROMATOGRAPHY	95.5% (MINIMUM)	99.9%
QUALITY CONTROL		JULY 2006
ACCEPTANCE DATE		

[redacted] Supervisor
Quality Control
Milwaukee, Wisconsin USA

N135-248 [redacted] 3-22-12

SIGMA-ALDRICH®

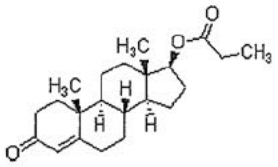
sigma-aldrich.com

3050 Spruce Street, Saint Louis, MO 63103, USA
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Testosterone propionate - solid

Product Number:	**T1875**
Lot Number:	**051M1803V**
Brand:	SIGMA
CAS Number:	57-85-2
MDL Number:	MFCD00003653
Formula:	C22H32O3
Formula Weight:	344.49 g/mol
Quality Release Date:	28 JUN 2011



Test	Specification	Result
Appearance (Color)	White to Off-White	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless to Faint Yellow	Very Faint Yellow
Solubility (Turbidity) 50 mg/mL, CHCl3	Clear	Clear
Infrared spectrum	Conforms to Structure	Conforms
Specific Rotation (+), C = 2 in dioxane at 25 deg C	82 - 87 °	85 °
Purity (HPLC)	≥ 98 %	102 %

[redacted]

[redacted] Manager
Analytical Services
St. Louis, Missouri US

Version Number: 1

Page 1 of 1

N135-248 7/5/12 [redacted] ~~7/3/12~~ @ [redacted] 7/5/12

SIGMA-ALDRICH®

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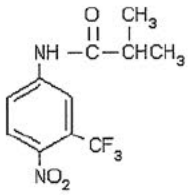
Website: www.sigmaaldrich.com
Email USA: techserv@sial.com
Outside USA: eurtechserv@sial.com

Certificate of Analysis

Product Name:
Flutamide

Product Number:	**F9397**
Lot Number:	**021M1406V**
Brand:	SIGMA
CAS Number:	13311-84-7
MDL Number:	MFCD00072009
Formula:	$C_{11}H_{11}F_3N_2O_3$
Formula Weight:	276.21 g/mol
Quality Release Date:	01 MAR 2011



Test	Specification	Result
Appearance (Color)	Yellow	Light Yellow
Appearance (Form)	Powder	Powder
Solubility (Color)	Yellow to Yellow-Green	Yellow - Green
Solubility (Turbidity) 50 mg/mL, EtOH	Clear to Hazy	Clear
Carbon	46.8 - 49.8%	48.0%
Nitrogen	9.8 - 10.4%	10.1%
Purity (TLC)	≥ 99%	100%

[redacted]

[redacted] Manager
Analytical Services
St. Louis, Missouri US

Version Number: 1

Page 1 of 1

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
Task Leader Date

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

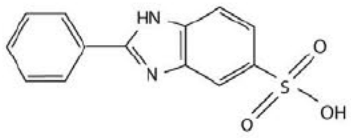
Dose Formulation Concentrations: 200 mg/mL; 64 mg/mL 20 mg/mL; 0 mg/mL

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

turning knowledge into practice

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%		
032312-B-01-2		180				
032312-B-01-3		196				
032312-B-02-1	200 (middle)	174	182 (8.1% RSD)	91.0%	189 (10.3% RSD)	94.5%
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%		
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%	56.6 (11.7% RSD)	88.4%
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%		
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%	17.8 (9.1% RSD)	89.0%
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%	59.3 (17.1% RSD)	92.7%
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%		
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%	18.5 (11.2% RSD)	92.5%
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%		
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]

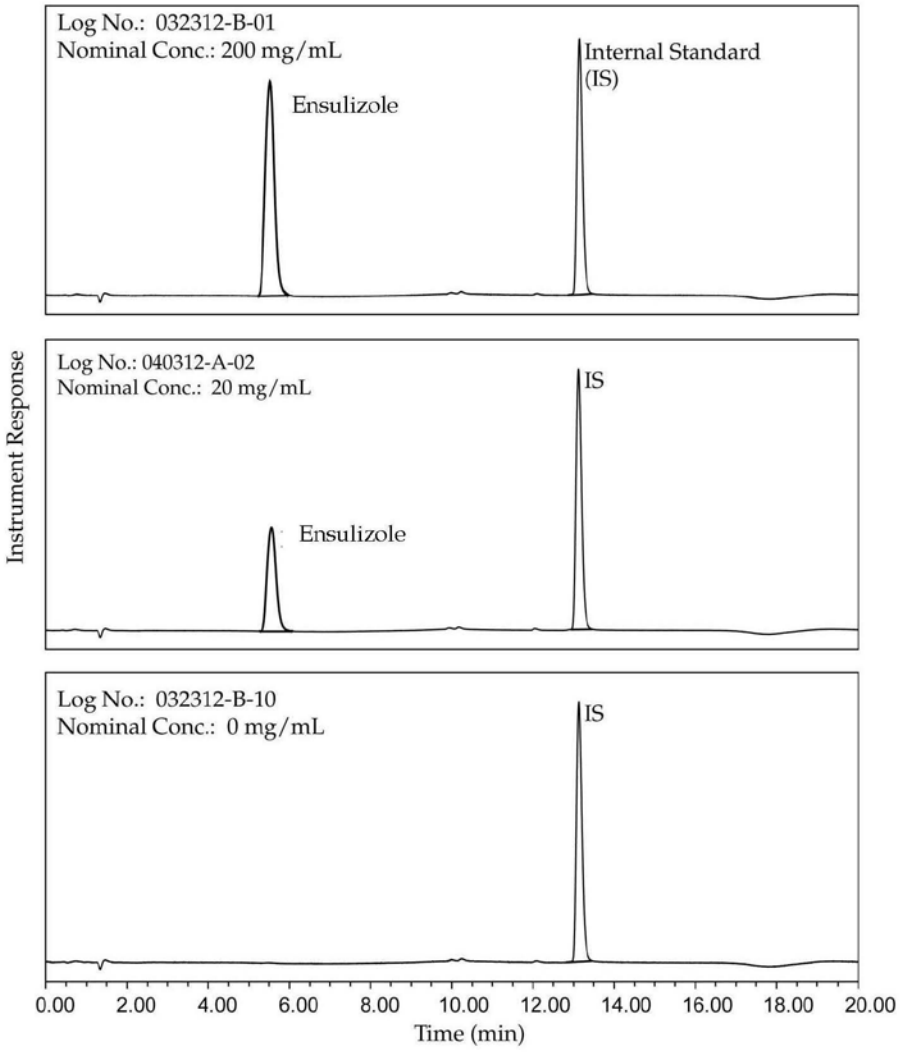


Figure 1. Representative Liquid Chromatograms of Ensulizole in Corn Oil

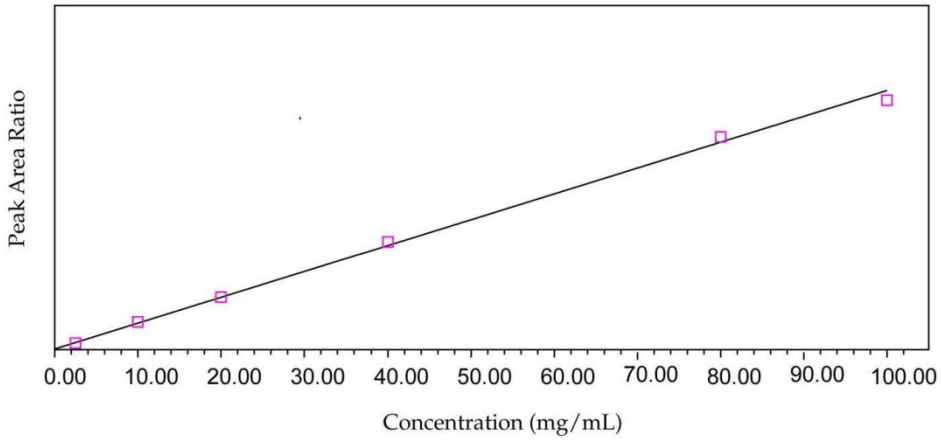


Figure 2. Plot of Vehicle Standards Data – Ensulizole in Corn Oil

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
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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

Prepared by:

[redacted]
Study Director

Approved by:

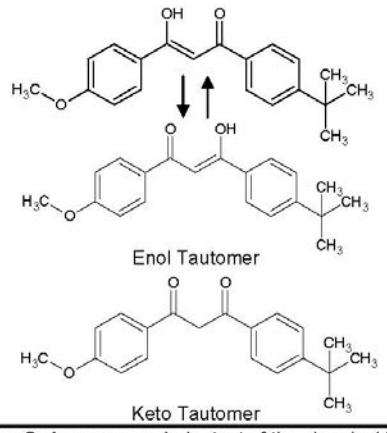
[redacted]
Joseph W. Algaier, Ph.D.
Principal Investigator

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$_3$	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 ~ 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$_3$	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)

[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.

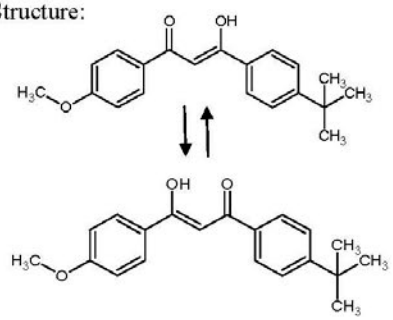
1. Introduction

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.

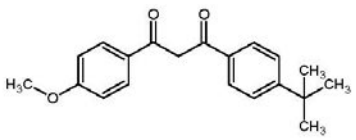
2. Chemical Information

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:

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

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)				

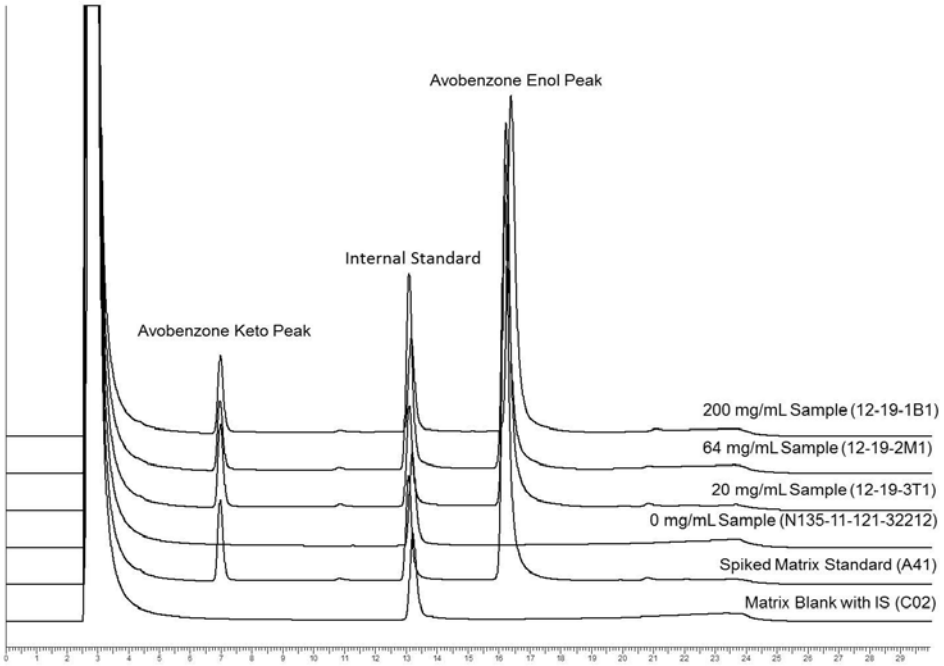


Figure 1. Representative HPLC/UV Chromatograms of Avobenzone in Corn Oil: Matrix Blank With IS, Spiked Matrix Standard, ILS, Inc., Prepared Formulation Samples at ~ 0, ~ 20, ~ 64, and ~ 200 mg/mL

MRIGlobal-NTP\Assignment_2236

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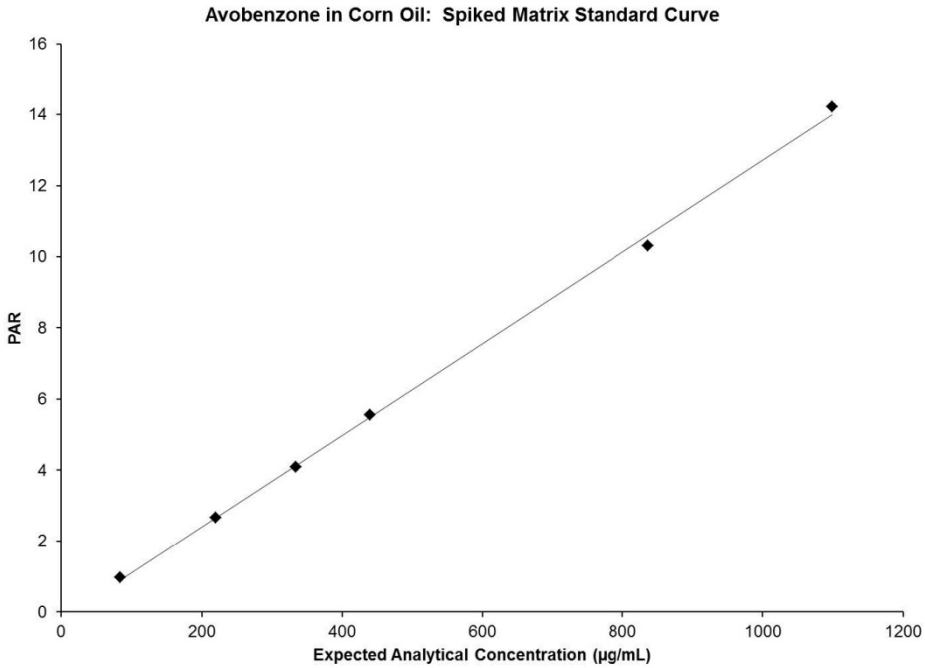

Figure 2. Avobenzone in Corn Oil Spiked Matrix Standard Curve: Analysis of ILS, Inc., Prepared Formulations

Appendix III:

Dose Times, Volumes and Dose Administration

Dose Administration:

					Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
1	001	Corn Oil Control	0	59	10:36	1.6	0.0	10:26	1.6	0.0	10:35	1.6	0.0	10:37	1.7	0.0	10:48	1.7	0.0
1	002			59	10:50	1.2	0.0	10:42	1.3	0.0	10:45	1.3	0.0	10:51	1.3	0.0	10:57	1.3	0.0
1	003			59	11:03	1.4	0.0	10:57	1.4	0.0	10:54	1.4	0.0	11:03	1.5	0.0	11:05	1.5	0.0
1	004			59	11:16	1.4	0.0	11:14	1.4	0.0	11:04	1.5	0.0	11:15	1.5	0.0	11:13	1.5	0.0
1	005			60	11:32	1.5	0.0	11:16	1.5	0.0	11:28	1.6	0.0	11:22	1.6	0.0	11:27	1.6	0.0
1	006			60	11:50	1.5	0.0	11:26	1.5	0.0	11:40	1.6	0.0	11:31	1.6	0.0	11:44	1.6	0.0
1	007			60	12:04	1.4	0.0	11:35	1.5	0.0	11:54	1.5	0.0	11:42	1.5	0.0	11:59	1.5	0.0
1	008			60	12:18	1.4	0.0	11:44	1.4	0.0	12:07	1.4	0.0	11:51	1.4	0.0	12:13	1.4	0.0

					Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
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 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	001	0.00	0	59	10:30	1.7	0.0	10:29	1.7	0.0	10:49	1.7	0.0	10:35	1.8	0.0	10:30	1.8	0.0	8:33	22:03
1	002			59	10:40	1.4	0.0	10:38	1.4	0.0	11:12	1.4	0.0	10:46	1.4	0.0	10:39	1.4	0.0	9:40	23:01
1	003			59	10:55	1.5	0.0	10:46	1.5	0.0	11:28	1.5	0.0	11:00	1.5	0.0	10:50	1.6	0.0	10:36	23:46
1	004			59	11:11	1.6	0.0	10:54	1.6	0.0	11:46	1.6	0.0	11:13	1.6	0.0	11:01	1.7	0.0	11:19	24:18
1	005			60	11:04	1.6	0.0	12:03	1.6	0.0	11:27	1.7	0.0	11:13	1.7	0.0	10:33	1.7	0.0	8:46	22:13
1	006			60	11:11	1.6	0.0	12:14	1.6	0.0	11:40	1.7	0.0	11:24	1.7	0.0	10:44	1.7	0.0	9:40	22:56
1	007			60	11:20	1.5	0.0	12:27	1.5	0.0	11:54	1.6	0.0	11:36	1.6	0.0	10:55	1.6	0.0	10:33	23:38
1	008			60	11:28	1.4	0.0	12:42	1.4	0.0	12:06	1.5	0.0	11:48	1.5	0.0	11:05	1.5	0.0	11:19	24:14

Dose Administration:

					Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
2	009	Ensulizole (320 mg/kg)	63.9	59	10:37	1.5	327.1	10:27	1.5	322.4	10:35	1.5	316.9	10:37	1.5	309.6	10:48	1.6	327.5
2	010			59	10:51	1.4	313.3	10:43	1.4	310.7	10:45	1.5	325.5	10:51	1.5	317.2	10:58	1.5	310.7
2	011			59	11:03	1.4	315.1	10:58	1.4	313.3	10:55	1.5	327.4	11:04	1.5	318.3	11:05	1.5	315.1
2	012			59	11:17	1.3	325.5	11:16	1.3	320.2	11:05	1.3	314.1	11:16	1.3	308.8	11:13	1.3	307.9
2	013			60	11:33	1.5	326.0	11:17	1.5	322.5	11:29	1.5	314.8	11:23	1.6	326.2	11:28	1.6	323.0
2	014			60	11:50	1.4	317.8	11:26	1.4	316.1	11:41	1.4	316.6	11:31	1.4	310.2	11:45	1.5	328.0
2	015			60	12:04	1.7	328.6	11:35	1.7	328.6	11:54	1.7	317.5	11:42	1.7	313.0	12:00	1.8	324.2
2	016			60	12:20	1.4	325.9	11:45	1.4	317.2	12:08	1.5	329.8	11:51	1.5	323.6	12:15	1.5	320.8

					Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
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 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	009	0.00	63.9	59	10:30	1.6	324.6	10:30	1.6	316.1	10:50	1.6	316.3	10:35	1.7	328.0	10:30	1.7	325.5	8:39	22:09
2	010			59	10:41	1.6	327.3	10:38	1.6	324.8	11:13	1.6	322.5	10:47	1.6	315.3	10:40	1.6	316.1	9:44	23:04
2	011			59	10:56	1.6	328.2	10:46	1.6	326.1	11:28	1.6	326.3	11:01	1.6	315.4	10:50	1.6	313.4	10:40	23:50
2	012			59	11:12	1.4	328.3	10:54	1.4	321.2	11:47	1.4	320.8	11:13	1.4	318.6	11:02	1.4	318.0	11:21	24:19
2	013			60	11:04	1.6	317.6	12:03	1.6	318.9	11:28	1.6	310.1	11:14	1.7	326.4	10:33	1.7	322.6	8:51	22:18
2	014			60	11:12	1.5	322.5	12:15	1.5	323.9	11:40	1.5	314.9	11:25	1.5	310.4	10:44	1.6	327.1	9:44	23:00
2	015			60	11:20	1.8	323.7	12:28	1.8	320.7	11:55	1.8	311.8	11:37	1.9	325.6	10:56	1.9	320.3	10:37	23:41
2	016			60	11:28	1.5	314.4	12:42	1.5	313.1	12:07	1.6	323.4	11:49	1.6	316.5	11:06	1.6	319.5	11:22	24:16

Dose Administration:

					Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
3	017	Ensulizole (1000 mg/kg)	189	59	10:38	1.4	947.0	10:28	1.4	943.3	10:36	1.4	919.7	10:38	1.5	965.3	10:49	1.5	970.9
3	018			59	10:52	1.6	944.4	10:44	1.6	930.7	10:46	1.7	970.4	10:52	1.7	952.3	10:58	1.7	932.1
3	019			59	11:04	1.4	942.6	10:59	1.4	956.6	10:55	1.4	940.6	11:05	1.4	920.0	11:06	1.5	977.6
3	020			59	11:17	1.4	968.5	11:17	1.4	970.7	11:05	1.4	952.8	11:16	1.4	926.1	11:14	1.4	920.0
3	021			60	11:35	1.3	953.8	11:17	1.3	943.9	11:29	1.3	935.3	11:23	1.3	925.1	11:28	1.3	914.7
3	022			60	11:51	1.5	936.0	11:27	1.5	929.8	11:41	1.6	965.2	11:32	1.6	959.1	11:47	1.6	948.3
3	023			60	12:05	1.4	942.3	11:36	1.4	941.0	11:55	1.4	917.5	11:43	1.4	914.0	12:01	1.5	969.2
3	024			60	12:20	1.5	922.9	11:45	1.5	927.1	12:09	1.6	952.4	11:52	1.6	947.4	12:16	1.6	943.5

					Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
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 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	017	0.00	189	59	10:31	1.5	944.1	10:30	1.5	936.0	10:51	1.5	933.8	10:36	1.6	969.2	10:31	1.6	957.9	8:44	22:13
3	018			59	10:42	1.8	962.9	10:39	1.8	958.9	11:14	1.8	950.0	10:47	1.8	920.7	10:41	1.9	962.5	9:48	23:07
3	019			59	10:56	1.5	964.3	10:47	1.5	956.2	11:29	1.5	961.7	11:01	1.5	932.3	10:51	1.5	927.7	10:42	23:51
3	020			59	11:13	1.4	914.3	10:55	1.5	965.9	11:48	1.5	960.4	11:14	1.5	939.7	11:02	1.5	931.0	11:25	24:23
3	021			60	11:05	1.4	970.3	12:04	1.3	912.0	11:28	1.4	954.9	11:14	1.4	947.0	10:34	1.4	939.0	8:55	22:21
3	022			60	11:12	1.6	932.8	12:15	1.6	937.1	11:41	1.7	970.4	11:26	1.7	957.1	10:45	1.7	950.9	9:49	23:04
3	023			60	11:20	1.5	953.9	12:29	1.5	951.7	11:55	1.5	940.6	11:38	1.5	934.4	10:56	1.5	931.3	10:40	23:44
3	024			60	11:29	1.6	931.3	12:43	1.7	970.1	12:07	1.7	956.0	11:50	1.7	943.9	11:06	1.7	941.4	11:25	24:19

Dose Administration:

					Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
4	025	Avobenzone (320 mg/kg)	58.91	59	10:39	1.3	296.9	10:29	1.3	294.7	10:37	1.3	289.5	10:39	1.4	301.6	10:49	1.4	297.5
4	026			59	10:53	1.3	285.3	10:45	1.4	300.3	10:46	1.4	297.7	10:53	1.4	290.0	10:58	1.4	285.2
4	027			59	11:05	1.6	298.4	11:00	1.6	297.2	10:56	1.6	295.7	11:05	1.6	286.1	11:07	1.7	298.7
4	028			59	11:18	1.5	297.8	11:18	1.5	293.8	11:06	1.5	294.6	11:17	1.5	286.2	11:15	1.5	285.2
4	029			60	11:36	1.4	290.5	11:18	1.4	288.4	11:30	1.5	299.2	11:23	1.5	302.6	11:30	1.5	296.9
4	030			60	11:52	1.5	304.4	11:27	1.5	300.4	11:42	1.5	293.2	11:33	1.5	294.8	11:47	1.5	290.6
4	031			60	12:08	1.4	299.4	11:36	1.4	293.7	11:56	1.4	288.3	11:43	1.4	284.6	12:02	1.4	284.8
4	032			60	12:20	1.5	298.5	11:46	1.5	296.3	12:09	1.5	289.9	11:52	1.5	290.6	12:16	1.5	286.8

					Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
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 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	025	0.00	58.91	59	10:32	1.4	290.2	10:31	1.5	304.6	10:53	1.4	291.9	10:36	1.5	293.9	10:31	1.5	294.6	8:52	22:21
4	026			59	10:44	1.5	303.3	10:39	1.5	304.5	11:14	1.4	289.0	10:48	1.5	300.1	10:41	1.5	293.4	9:52	23:11
4	027			59	10:57	1.7	293.9	10:47	1.7	290.7	11:30	1.7	288.3	11:02	1.8	297.8	10:51	1.8	294.1	10:45	23:54
4	028			59	11:14	1.5	285.5	10:56	1.6	299.6	11:49	1.6	299.2	11:15	1.6	297.3	11:03	1.6	296.5	11:29	24:26
4	029			60	11:06	1.5	297.4	12:05	1.5	295.3	11:29	1.5	294.5	11:15	1.5	292.0	10:34	1.5	290.1	8:59	22:25
4	030			60	11:13	1.5	286.9	12:16	1.5	292.0	11:42	1.6	303.9	11:27	1.6	299.4	10:45	1.6	294.6	9:53	23:08
4	031			60	11:21	1.5	297.0	12:30	1.5	294.9	11:56	1.5	294.1	11:38	1.5	289.1	10:57	1.5	286.3	10:44	23:47
4	032			60	11:29	1.6	297.4	12:44	1.6	297.9	12:08	1.6	292.9	11:50	1.6	293.9	11:07	1.6	291.9	11:28	24:21

Dose Administration:

					Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
5	033	Avobenzone (1000 mg/kg)	209.49	59	10:40	1.6	1055.4	10:30	1.6	1075.3	10:37	1.6	1065.1	10:40	1.6	1049.4	10:50	1.6	1030.4
5	034			59	10:53	1.4	1061.9	10:46	1.4	1060.3	10:47	1.4	1046.0	10:53	1.4	1040.4	10:59	1.4	1021.2
5	035			59	11:06	1.4	1045.2	11:02	1.4	1046.0	10:57	1.4	1029.1	11:06	1.4	1013.4	11:07	1.5	1068.8
5	036			59	11:19	1.4	1041.9	11:19	1.4	1041.9	11:07	1.4	1031.2	11:18	1.5	1082.8	11:15	1.5	1064.1
5	037			60	11:38	1.6	1079.8	11:19	1.6	1080.5	11:31	1.6	1046.1	11:24	1.6	1019.4	11:31	1.7	1062.1
5	038			60	11:53	1.5	1047.8	11:28	1.5	1037.4	11:43	1.5	1017.3	11:33	1.6	1057.4	11:48	1.6	1053.4
5	039			60	12:08	1.4	1073.5	11:37	1.3	1010.5	11:57	1.4	1030.2	11:44	1.4	1023.7	12:03	1.4	1025.1
5	040			60	12:22	1.3	1037.9	11:46	1.3	1041.4	12:10	1.3	1031.2	11:53	1.3	1028.1	12:18	1.3	1012.8

					Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
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 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	033	Avobenzone (1000 mg/kg)	209.49	59	10:32	1.7	1072.4	10:31	1.7	1048.4	10:54	1.7	1054.0	10:37	1.7	1028.4	10:32	1.8	1073.4	8:57	22:25
5	034			59	10:45	1.4	1013.4	10:40	1.4	1011.7	11:15	1.5	1081.3	10:49	1.5	1054.1	10:42	1.5	1040.2	9:57	23:15
5	035			59	10:58	1.5	1055.5	10:48	1.5	1028.9	11:31	1.5	1048.5	11:02	1.5	1032.0	10:52	1.5	1022.6	10:49	23:57
5	036			59	11:15	1.5	1050.3	10:56	1.5	1034.7	11:50	1.5	1038.8	11:16	1.6	1078.5	11:04	1.6	1068.8	11:31	24:27
5	037			60	11:06	1.7	1064.4	12:05	1.7	1051.8	11:30	1.7	1032.0	11:15	1.7	1018.4	10:35	1.8	1077.1	9:04	22:29
5	038			60	11:13	1.6	1062.1	12:17	1.6	1051.7	11:43	1.7	1073.3	11:27	1.7	1056.5	10:46	1.7	1050.5	9:58	23:12
5	039			60	11:21	1.4	1028.4	12:31	1.4	1012.4	11:56	1.5	1068.5	11:39	1.5	1062.3	10:57	1.5	1045.0	10:48	23:51
5	040			60	11:29	1.3	1018.5	12:44	1.3	1018.5	12:09	1.4	1077.9	11:52	1.4	1063.8	11:08	1.4	1081.0	11:32	24:24

Dose Administration:

						Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
6	041	Corn Oil Control (0 mg/kg) + TP (0.4 mg/kg)	Gavage	0	59	10:41	1.5	0.0	10:31	1.5	0.0	10:38	1.6	0.0	10:41	1.6	0.0	10:50	1.7	0.0
			Subcutaneous				0.15			0.15			0.16			0.16			0.17	
6	042		Gavage	0	59	10:54	1.3	0.0	10:48	1.3	0.0	10:48	1.3	0.0	10:55	1.4	0.0	10:59	1.4	0.0
			Subcutaneous				0.13			0.13			0.13			0.14			0.14	
6	043		Gavage	0	59	11:07	1.4	0.0	11:04	1.4	0.0	10:57	1.3	0.0	11:07	1.5	0.0	11:08	1.5	0.0
			Subcutaneous				0.14			0.14			0.13			0.15			0.15	
6	044		Gavage	0	59	11:19	1.5	0.0	11:20	1.5	0.0	11:07	1.5	0.0	11:19	1.6	0.0	11:16	1.6	0.0
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	
6	045		Gavage	0	60	11:40	1.5	0.0	11:20	1.5	0.0	11:32	1.5	0.0	11:25	1.6	0.0	11:32	1.6	0.0
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	
6	046		Gavage	0	60	11:54	1.5	0.0	11:29	1.5	0.0	11:44	1.5	0.0	11:35	1.6	0.0	11:49	1.6	0.0
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	
6	047		Gavage	0	60	12:10	1.4	0.0	11:38	1.4	0.0	11:58	1.5	0.0	11:45	1.5	0.0	12:04	1.6	0.0
			Subcutaneous				0.14			0.14			0.15			0.15			0.16	
6	048		Gavage	0	60	12:23	1.4	0.0	11:47	1.5	0.0	12:12	1.5	0.0	11:53	1.6	0.0	12:19	1.6	0.0
			Subcutaneous				0.14			0.15			0.15			0.16			0.16	

						Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 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	041	0.00	Gavage	0	59	10:33	1.7	0.0	10:32	1.7	0.0	10:58	1.8	0.0	10:38	1.8	0.0	10:33	1.8	0.0	9:02	22:29
			Subcutaneous				0.17			0.17			0.18			0.18			0.18			
6	042		Gavage	0	59	10:45	1.5	0.0	10:40	1.5	0.0	11:16	1.5	0.0	10:50	1.5	0.0	10:43	1.6	0.0	10:03	23:20
			Subcutaneous				0.15			0.15			0.15			0.15			0.16			
6	043		Gavage	0	59	11:01	1.5	0.0	10:48	1.6	0.0	11:33	1.6	0.0	11:04	1.6	0.0	10 53	1.6	0.0	10 51	23:58
			Subcutaneous				0.15			0.16			0.16			0.16			0.16			
6	044		Gavage	0	59	11:16	1.7	0.0	10 57	1.7	0.0	11:52	1.7	0.0	11:17	1.8	0.0	11:05	1.8	0.0	11:33	24:28
			Subcutaneous				0.17			0.17			0.17			0.18			0.18			
6	045		Gavage	0	60	11:06	1.7	0.0	12:06	1.7	0.0	11:31	1.7	0.0	11:16	1.8	0.0	10:36	1.8	0.0	9:07	22:31
			Subcutaneous				0.17			0.17			0.17			0.18			0.18			
6	046		Gavage	0	60	11:14	1.7	0.0	12:18	1.7	0.0	11:44	1.7	0.0	11:28	1.8	0.0	10:47	1.8	0.0	10:01	23:14
			Subcutaneous				0.17			0.17			0.17			0.18			0.18			
6	047		Gavage	0	60	11:22	1.6	0.0	12:32	1.7	0.0	11:58	1.7	0.0	11:40	1.8	0.0	10 58	1.8	0.0	10 51	23:53
			Subcutaneous				0.16			0.17			0.17			0.18			0.18			
6	048		Gavage	0	60	11:30	1.6	0.0	12:47	1.6	0.0	12:10	1.7	0.0	11:53	1.7	0.0	11:09	1.7	0.0	11:36	24:27
			Subcutaneous				0.16			0.16			0.17			0.17			0.17			

Dose Administration:

						Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
7	049	Ensulizole (100 mg/kg) + TP (0.4 mg/kg)	Gavage	18.5	59	10:42	1.3	90.1	10:32	1.4	94.9	10:39	1.4	93.4	10:42	1.4	89.7	10:52	1.5	94.2
			Subcutaneous				0.13			0.14			0.14			0.14			0.15	
7	050		Gavage	18.5	59	10:55	1.5	90.1	10:49	1.5	90.2	10:49	1.6	94.5	10:56	1.6	91.1	11:00	1.7	93.7
			Subcutaneous				0.15			0.15			0.16			0.16			0.17	
7	051		Gavage	18.5	59	11:08	1.3	90.6	11:05	1.3	90.6	10:58	1.4	93.5	11:08	1.4	89.8	11:09	1.5	93.4
			Subcutaneous				0.13			0.13			0.14			0.14			0.15	
7	052		Gavage	18.5	59	11:21	1.5	93.2	11:22	1.5	91.1	11:08	1.6	94.9	11:20	1.6	92.6	11:17	1.6	89.8
			Subcutaneous				0.15			0.15			0.16			0.16			0.16	
7	053		Gavage	18.5	60	11:41	1.5	92.7	11:20	1.5	91.9	11:33	1.6	94.2	11:26	1.6	91.1	11:33	1.7	93.7
			Subcutaneous				0.15			0.15			0.16			0.16			0.17	
7	054		Gavage	18.5	60	11:55	1.5	95.6	11:29	1.5	94.0	11:45	1.5	91.4	11:36	1.6	95.4	11:50	1.6	91.4
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	
7	055		Gavage	18.5	60	12:10	1.5	93.2	11:39	1.5	93.3	12:00	1.6	95.4	11:45	1.6	94.4	12:04	1.6	91.4
			Subcutaneous				0.15			0.15			0.16			0.16			0.16	
7	056		Gavage	18.5	60	12:55	1.4	93.2	11:48	1.4	92.7	12:13	1.4	89.8	11:54	1.5	95.2	12:20	1.5	91.9
			Subcutaneous				0.14			0.14			0.14			0.15			0.15	

						Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 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	049	0.00	Gavage	18.5	59	10:34	1.5	91.4	10:33	1.6	95.3	11:01	1.6	94.9	10:39	1.6	90.7	10:34	1.7	94 9	9:06	22:32
			Subcutaneous				0.15			0.16			0.16			0.16			0.17			
7	050		Gavage	18.5	59	10:46	1.7	92.0	10:41	1.8	94.2	11:18	1.7	91.3	10:51	1.8	93.1	10:44	1.8	91.0	10:07	23:23
			Subcutaneous				0.17			0.18			0.17			0.18			0.18			
7	051		Gavage	18.5	59	11:02	1.5	90.5	10:49	1.6	93.3	11:34	1.6	94.6	11:05	1.6	90.5	10 54	1.7	91 9	10 55	24:01
			Subcutaneous				0.15			0.16			0.16			0.16			0.17			
7	052		Gavage	18.5	59	11:17	1.7	92.9	10 58	1.7	90.0	11:53	1.8	95.1	11:19	1.8	92.3	11:06	1.9	94 9	11:37	24:31
			Subcutaneous				0.17			0.17			0.18			0.18			0.19			
7	053		Gavage	18.5	60	11:07	1.7	91.1	12:07	1.8	94.5	11:32	1.8	91.5	11:17	1.9	94.4	10:37	1.9	94 2	9:12	22:35
			Subcutaneous				0.17			0.18			0.18			0.19			0.19			
7	054		Gavage	18.5	60	11:15	1.6	90.5	12:19	1.7	94.6	11:45	1.7	91.2	11:29	1.8	94.7	10:48	1.8	92 2	10:06	23:18
			Subcutaneous				0.16			0.17			0.17			0.18			0.18			
7	055		Gavage	18.5	60	11:23	1.7	95.1	12:33	1.7	94.0	11:59	1.7	91.2	11:41	1.7	90.5	10 59	1.8	94 3	10 54	23:55
			Subcutaneous				0.17			0.17			0.17			0.17			0.18			
7	056		Gavage	18.5	60	11:31	1.5	91.1	12:49	1.5	89.7	12:11	1.6	94.1	11:54	1.6	92.2	11:10	1.6	90 9	11:39	24:29
			Subcutaneous				0.15			0.15			0.16			0.16			0.16			

Dose Administration:

						Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
8	057	Ensulizole (320 mg/kg) + TP (0.4 mg/kg)	Gavage	63.9	59	10:44	1.3	323.9	10:33	1.3	317.9	10:40	1.4	329.3	10:44	1.4	321.8	10:53	1.4	310.0
			Subcutaneous				0.13			0.13			0.14			0.14			0.14	
8	058		Gavage	63.9	59	10:56	1.6	328.9	10:50	1.6	325.4	10:50	1.6	314.8	10:57	1.7	318.9	11:01	1.7	310.9
			Subcutaneous				0.16			0.16			0.16			0.17			0.17	
8	059		Gavage	63.9	59	11:09	1.4	308.8	11:07	1.5	324.6	10:59	1.5	317.0	11:09	1.6	328.9	11:10	1.6	317.9
			Subcutaneous				0.14			0.15			0.15			0.16			0.16	
8	060		Gavage	63.9	59	11:22	1.4	320.3	11:24	1.4	312.1	11:09	1.5	325.7	11:21	1.5	321.9	11:17	1.6	325.9
			Subcutaneous				0.14			0.14			0.15			0.15			0.16	
8	061		Gavage	63.9	60	11:42	1.5	328.8	11:21	1.5	319.3	11:34	1.5	316.5	11:26	1.6	326.7	11:34	1.6	315.8
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	
8	062		Gavage	63.9	60	11:56	1.5	320.2	11:30	1.5	314.5	11:46	1.6	319.4	11:37	1.6	314.7	11:53	1.7	328.2
			Subcutaneous				0.15			0.15			0.16			0.16			0.17	
8	063		Gavage	63.9	60	12:11	1.4	312.7	11:40	1.5	324.5	12:01	1.5	312.8	11:46	1.6	328.0	12:06	1.6	323.2
			Subcutaneous				0.14			0.15			0.15			0.16			0.16	
8	064		Gavage	63.9	60	12:26	1.4	328.4	11:49	1.4	317.5	12:15	1.5	324.0	11:55	1.5	314.7	12:21	1.6	320.4
			Subcutaneous				0.14			0.14			0.15			0.15			0.16	

						Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 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	057	Ensulizole (320 mg/kg) + TP (0.4 mg/kg)	Gavage	63 9	59	10:35	1.5	319.5	10:34	1.5	310.5	11:03	1.6	322.8	10:40	1.6	313.0	10:34	1.7	325.5	9:11	22:37
			Subcutaneous				0.15			0.15			0.16			0.16			0.17			
8	058		Gavage	63 9	59	10:47	1.8	318.6	10:42	1.8	311.7	11:20	1.9	323.2	10:53	2.0	323.2	10:45	2.0	318.8	10:12	23:27
			Subcutaneous				0.18			0.18			0.19			0.20			0.20			
8	059		Gavage	63 9	59	11:03	1.7	328.0	10 50	1.7	321.1	11:36	1.7	322.2	11:06	1.8	322.1	10 55	1.8	314.3	10 58	24:03
			Subcutaneous				0.17			0.17			0.17			0.18			0.18			
8	060		Gavage	63 9	59	11:18	1.6	320.3	10 58	1.7	329.0	11:54	1.7	323.9	11:20	1.7	313.5	11:07	1.8	324.4	11:40	24:33
			Subcutaneous				0.16			0.17			0.17			0.17			0.18			
8	061		Gavage	63 9	60	11:08	1.6	313.2	12:08	1.7	326.2	11:33	1.7	317.8	11:18	1.7	312.3	10:38	1.8	322.9	9:16	22:38
			Subcutaneous				0.16			0.17			0.17			0.17			0.18			
8	062		Gavage	63 9	60	11:15	1.7	323.0	12:20	1.7	313.9	11:46	1.8	323.8	11:30	1.8	317.1	10:49	1.8	311.5	10:10	23:21
			Subcutaneous				0.17			0.17			0.18			0.18			0.18			
8	063		Gavage	63 9	60	11:24	1.6	317.2	12:34	1.6	312.9	12:00	1.7	325.5	11:42	1.7	318.8	11:00	1.7	314.7	10 58	23:58
			Subcutaneous				0.16			0.16			0.17			0.17			0.17			
8	064		Gavage	63 9	60	11:32	1.6	310.9	12 50	1.7	324.2	12:12	1.7	314.3	11:55	1.8	323.5	11:11	1.8	316.0	11:42	24:31
			Subcutaneous				0.16			0.17			0.17			0.18			0.18			

Dose Administration:

						Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
9	065	Ensulizole (1000 mg/kg) + TP (0.4 mg/kg)	Gavage	189	59	10:45	1.5	953.6	10:34	1.5	941.5	10:41	1.5	928.6	10:45	1.6	946.2	10:54	1.6	916.6
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	
9	066		Gavage	189	59	10:57	1.5	948.8	10:52	1.5	936.0	10:50	1.5	922.9	10:58	1.6	948.3	11:01	1.6	929.9
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	
9	067		Gavage	189	59	11:10	1.4	956.3	11:08	1.4	943.7	11:00	1.4	916.8	11:10	1.5	948.5	11:10	1.5	932.3
			Subcutaneous				0.14			0.14			0.14			0.15			0.15	
9	068		Gavage	189	59	11:23	1.3	912.4	11:25	1.4	962.9	11:10	1.4	936.0	11:22	1.5	966.3	11:18	1.5	940.0
			Subcutaneous				0.13			0.14			0.14			0.15			0.15	
9	069		Gavage	189	60	11:43	1.4	940.0	11:22	1.4	916.8	11:35	1.5	933.2	11:26	1.6	953.6	11:36	1.6	917.8
			Subcutaneous				0.14			0.14			0.15			0.16			0.16	
9	070		Gavage	189	60	11:58	1.3	915.8	11:31	1.4	963.2	11:48	1.4	920.0	11:37	1.5	955.8	11:54	1.5	931.6
			Subcutaneous				0.13			0.14			0.14			0.15			0.15	
9	071		Gavage	189	60	12:13	1.5	930.4	11:40	1.5	926.2	12:02	1.6	960.9	11:47	1.6	950.6	12:07	1.7	973.3
			Subcutaneous				0.15			0.15			0.16			0.16			0.17	
9	072		Gavage	189	60	12:27	1.5	966.6	11:50	1.5	958.7	12:17	1.5	923.2	11:56	1.6	973.3	12:22	1.6	956.7
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	

						Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 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	065	Ensulizole (1000 mg/kg) + TP (0.4 mg/kg)	Gavage	189	59	10:35	1.7	951.4	10:34	1.7	922.5	11:04	1.8	962.4	10:41	1.8	936.7	10:35	1.9	959.1	9:17	22:42
			Subcutaneous				0.17			0.17			0.18			0.18			0.19			
9	066		Gavage	189	59	10:48	1.7	960.8	10:42	1.7	932.4	11:21	1.7	923.0	10:54	1.8	957.0	10:46	1.8	934.1	10:15	23:29
			Subcutaneous				0.17			0.17			0.17			0.18			0.18			
9	067		Gavage	189	59	11:04	1.6	974.2	10:50	1.6	966.4	11:40	1.6	965.8	11:07	1.6	933.6	10:56	1.6	927.3	11:02	24:06
			Subcutaneous				0.16			0.16			0.16			0.16			0.16			
9	068		Gavage	189	59	11:19	1.6	972.7	10:59	1.6	938.5	11:55	1.6	933.6	11:21	1.7	958.5	11:08	1.7	937.6	11:43	24:35
			Subcutaneous				0.16			0.16			0.16			0.17			0.17			
9	069		Gavage	189	60	11:08	1.7	953.7	12:09	1.7	932.7	11:34	1.8	944.2	11:19	1.8	926.0	10:39	1.9	950.8	9:20	22:41
			Subcutaneous				0.17			0.17			0.18			0.18			0.19			
9	070		Gavage	189	60	11:16	1.5	914.8	12:22	1.6	968.0	11:47	1.6	937.4	11:31	1.7	972.5	10:50	1.7	954.5	10:14	23:24
			Subcutaneous				0.15			0.16			0.16			0.17			0.17			
9	071		Gavage	189	60	11:24	1.7	946.4	12:35	1.7	946.4	12:01	1.8	966.8	11:43	1.8	942.6	11:01	1.8	928.5	11:01	24:00
			Subcutaneous				0.17			0.17			0.18			0.18			0.18			
9	072		Gavage	189	60	11:33	1.6	940.9	12:51	1.6	958.2	12:13	1.7	969.2	11:56	1.7	961.7	11:12	1.7	946.9	11:46	24:34
			Subcutaneous				0.16			0.16			0.17			0.17			0.17			

Dose Administration:

						Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
10	073	Avobenzone (100 mg/kg) + TP (0.4 mg/kg)	Gavage	20.688	59	10:46	1.4	104.0	10:37	1.4	102.4	10:42	1.5	107.0	10:47	1.5	104.1	10:54	1.5	100.5
			Subcutaneous				0.14			0.14			0.15			0.15			0.15	
10	074		Gavage	20.688	59	10:58	1.5	101.8	10:53	1.5	100.5	10 51	1.6	104.3	10:59	1.7	106.2	11:02	1.7	104.8
			Subcutaneous				0.15			0.15			0.16			0.17			0.17	
10	075		Gavage	20.688	59	11:12	1.4	105.5	11:10	1.4	103.7	11:01	1.4	101.0	11:11	1.5	104.3	11:11	1.5	101.5
			Subcutaneous				0.14			0.14			0.14			0.15			0.15	
10	076		Gavage	20.688	59	11:24	1.4	106.6	11:26	1.4	104.9	11:11	1.4	101.6	11:23	1.5	103.8	11:19	1.5	102.2
			Subcutaneous				0.14			0.14			0.14			0.15			0.15	
10	077		Gavage	20.688	60	11:46	1.5	105.3	11:23	1.5	103.4	11:36	1.6	105.9	11:28	1.6	103.3	11:38	1.7	106.4
			Subcutaneous				0.15			0.15			0.16			0.16			0.17	
10	078		Gavage	20.688	60	11:59	1.6	105.4	11:32	1.6	102.1	11:49	1.7	105.5	11:38	1.7	102.1	11:55	1.8	102.0
			Subcutaneous				0.16			0.16			0.17			0.17			0.18	
10	079		Gavage	20.688	60	12:14	1.4	105.7	11:41	1.4	103.4	12:03	1.5	106.4	11:48	1.5	104.0	12:08	1.6	106.7
			Subcutaneous				0.14			0.14			0.15			0.15			0.16	
10	080		Gavage	20.688	60	10:28	1.5	106.0	11:51	1.5	104.0	12:18	1.5	100.6	11:56	1.6	103.6	12:23	1.6	101.3
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	

						Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 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
10	073	0.00	Gavage	20.688	59	10:36	1.6	104.5	10:35	1.6	102.9	11:06	1.7	106.4	10:42	1.7	104.4	10:36	1.7	102.9	9:23	22:47
			Subcutaneous				0.16			0.16			0.17			0.17			0.17			
10	074		Gavage	20.688	59	10:49	1.7	102.0	10:43	1.7	101.8	11:22	1.8	104.5	10 55	1.8	100.8	10:46	1.9	105.6	10:19	23:33
			Subcutaneous				0.17			0.17			0.18			0.18			0.19			
10	075		Gavage	20.688	59	11:06	1.6	105.8	10:52	1.6	104.4	11:41	1.6	102.9	11:08	1.7	105.6	10:57	1.7	105.5	11:05	24:08
			Subcutaneous				0.16			0.16			0.16			0.17			0.17			
10	076		Gavage	20.688	59	11:20	1.6	104.8	11:01	1.6	102.3	11 57	1.7	106.0	11:22	1.7	104.1	11:09	1.7	102.4	11:47	24:38
			Subcutaneous				0.16			0.16			0.17			0.17			0.17			
10	077		Gavage	20.688	60	11:09	1.7	104.5	12:10	1.7	103.2	11:35	1.7	101.0	11:20	1.8	104.2	10:40	1.8	101.4	9:26	22:46
			Subcutaneous				0.17			0.17			0.17			0.18			0.18			
10	078		Gavage	20.688	60	11:17	1.8	102.4	12:23	1.9	106.0	11:48	1.9	102.1	11:32	2.0	105.4	10:51	2.0	103.4	10:18	23:27
			Subcutaneous				0.18			0.19			0.19			0.20			0.20			
10	079		Gavage	20.688	60	11:25	1.6	105.3	12:36	1.6	103.8	12:02	1.6	100.5	11:44	1.6	100.9	11:02	1.7	105.0	11:05	24:03
			Subcutaneous				0.16			0.16			0.16			0.16			0.17			
10	080		Gavage	20.688	60	11:33	1.7	104.5	12:52	1.7	103.3	12:14	1.7	102.1	11 57	1.7	101.9	11:13	1.8	104.0	11:49	24:36
			Subcutaneous				0.17			0.17			0.17			0.17			0.18			

Dose Administration:

						Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
11	081	Avobenzone (320 mg/kg) + TP (0.4 mg/kg)	Gavage	58.910	59	10:47	1.5	302.3	10:38	1.5	302.0	10:42	1.5	294.8	10:48	1.6	299.0	10:54	1.6	294.4
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	
11	082		Gavage	58.910	59	10:59	1.5	286.4	10:54	1.5	285.8	10:52	1.6	299.7	11:00	1.7	302.4	11:03	1.7	299.5
			Subcutaneous				0.15			0.15			0.16			0.17			0.17	
11	083		Gavage	58.910	59	11:12	1.4	299.7	11:11	1.4	293.5	11:02	1.4	285.4	11:12	1.5	297.3	11:12	1.5	288.6
			Subcutaneous				0.14			0.14			0.14			0.15			0.15	
11	084		Gavage	58.910	59	11:25	1.4	301.9	11:28	1.4	298.5	11:12	1.4	290.3	11:24	1.4	284.6	11:20	1.5	297.8
			Subcutaneous				0.14			0.14			0.14			0.14			0.15	
11	085		Gavage	58.910	60	11:46	1.4	302.0	11:24	1.4	299.6	11:37	1.5	304.1	11:29	1.5	297.0	11:40	1.5	289.2
			Subcutaneous				0.14			0.14			0.15			0.15			0.15	
11	086		Gavage	58.910	60	12:00	1.5	298.8	11:33	1.5	293.8	11:50	1.6	303.4	11:39	1.6	291.7	11:56	1.7	299.2
			Subcutaneous				0.15			0.15			0.16			0.16			0.17	
11	087		Gavage	58.910	60	12:16	1.5	291.8	11:42	1.6	303.3	12:04	1.6	291.1	11:49	1.6	286.1	12:10	1.7	295.3
			Subcutaneous				0.15			0.16			0.16			0.16			0.17	
11	088		Gavage	58.910	60	10:29	1.3	285.2	11:52	1.4	302.7	12:19	1.4	291.2	11:57	1.5	303.6	12:24	1.5	297.8
			Subcutaneous				0.13			0.14			0.14			0.15			0.15	

						Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 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
11	081	0.00	Gavage	58.910	59	10:38	1.6	286.6	10:35	1.7	295.1	11:07	1.7	293.0	10:43	1.8	300.7	10:37	1.8	295.7	9:26	22:49
			Subcutaneous				0.16			0.17			0.17			0.18			0.18			
11	082		Gavage	58.910	59	10:50	1.7	291.4	10:44	1.8	300.2	11:23	1.8	299.0	10:57	1.8	289.2	10:47	1.9	297.4	10:23	23:36
			Subcutaneous				0.17			0.18			0.18			0.18			0.19			
11	083		Gavage	58.910	59	11:07	1.6	298.2	10:52	1.6	289.9	11:42	1.6	292.9	11:09	1.7	296.3	10:58	1.7	293.3	11:09	24:11
			Subcutaneous				0.16			0.16			0.16			0.17			0.17			
11	084		Gavage	58.910	59	11:21	1.5	291.4	11:02	1.6	302.6	11:59	1.6	301.9	11:24	1.6	290.6	11:10	1.7	303.0	11:51	24:41
			Subcutaneous				0.15			0.16			0.16			0.16			0.17			
11	085		Gavage	58.910	60	11:10	1.5	290.4	12:11	1.5	287.4	11:36	1.6	295.9	11:21	1.6	291.8	10:41	1.6	286.5	9:29	22:48
			Subcutaneous				0.15			0.15			0.16			0.16			0.16			
11	086		Gavage	58.910	60	11:18	1.7	294.7	12:25	1.7	291.0	11:50	1.8	295.4	11:33	1.8	295.9	10:52	1.8	289.2	10:22	23:30
			Subcutaneous				0.17			0.17			0.18			0.18			0.18			
11	087		Gavage	58.910	60	11:26	1.7	288.8	12:38	1.7	288.0	12:03	1.8	295.7	11:45	1.8	290.6	11:03	1.8	288.4	11:09	24:06
			Subcutaneous				0.17			0.17			0.18			0.18			0.18			
11	088		Gavage	58.910	60	11:34	1.5	294.0	12:53	1.5	291.7	12:15	1.6	302.0	11:58	1.6	295.3	11:14	1.6	294.2	11:53	24:39
			Subcutaneous				0.15			0.15			0.16			0.16			0.16			

Dose Administration:

						Day 1 (6 & 7 April 2012)			Day 2 (7 & 8 April 2012)			Day 3 (8 & 9 April 2012)			Day 4 (9 & 10 April 2012)			Day 5 (10 & 11 April 2012)		
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)
12	089	Avobenzone (1000 mg/kg) + TP (0.4 mg/kg)	Gavage	209.49	59	10:48	1.4	1075.1	10:39	1.4	1065.7	10:43	1.4	1054.6	10:49	1.5	1078.0	10:56	1.5	1045.7
			Subcutaneous				0.14			0.14			0.14			0.15			0.15	
12	090		Gavage	209.49	59	11:01	1.3	1009.8	10:55	1.3	1015.4	10 53	1.3	1015.8	11:01	1.4	1051.6	11:04	1.4	1034.9
			Subcutaneous				0.13			0.13			0.13			0.14			0.14	
12	091		Gavage	209.49	59	11:14	1.5	1019.3	11:12	1.5	1026 2	11:03	1.6	1049.7	11:13	1.6	1020.3	11:12	1.7	1062.1
			Subcutaneous				0.15			0.15			0.16			0.16			0.17	
12	092		Gavage	209.49	59	11:27	1.5	1072.5	11:29	1.5	1065 9	11:13	1.5	1027.9	11:25	1.6	1064.1	11:20	1.6	1025.3
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	
12	093		Gavage	209.49	60	11:48	1.4	1047.1	11:24	1.4	1056.1	11:38	1.4	1019.8	11:29	1.4	1066.5	11:41	1 5	1072.5
			Subcutaneous				0.14			0.14			0.14			0.14			0.15	
12	094		Gavage	209.49	60	12:01	1.3	1022.7	11:33	1.4	1084.6	11 52	1.4	1067.3	11:41	1.3	1033.1	11:57	1.4	1039.3
			Subcutaneous				0.13			0..14			0.14			0.13			0.14	
12	095		Gavage	209.49	60	12:17	1.6	1080.5	11:43	1 5	1016 3	12:05	1.6	1069.5	11:50	1.6	1066.1	12:11	1.6	1040.6
			Subcutaneous				0.16			0.15			0.16			0.16			0.16	
12	096		Gavage	209.49	60	12:30	1.5	1066.3	11:52	1 5	1035.4	12:20	1.5	1019.9	11:58	1.6	1072.2	12:25	1.6	1029.1
			Subcutaneous				0.15			0.15			0.15			0.16			0.16	

						Day 6 (11 & 12 April 2012)			Day 7 (12 & 13 April 2012)			Day 8 (13 & 14 April 2012)			Day 9 (14 & 15 April 2012)			Day 10 (15 & 16 April 2012)			Day 11 (16 & 17 April 2012)	
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 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
12	089	0.00	Gavage	209.49	59	10:39	1.5	1031.3	10:37	1.6	1070.2	11:10	1.6	1075.7	10:44	1.6	1051.1	10:38	1.6	1030.4	9:31	22 53
			Subcutaneous				0.15			0.16			0.16			0.16			0.16			
12	090		Gavage	209.49	59	10:52	1.4	1015.9	10:44	1.5	1071.4	11:24	1.4	1023.3	10 58	1 5	1057.7	10:48	1 5	1028.9	10:27	23:39
			Subcutaneous				0.14			0.15			0.14			0.15			0.15			
12	091		Gavage	209.49	59	11:09	1.7	1030.2	10:53	1.8	1070.6	11:43	1.8	1049.8	11:10	1 9	1069.7	10:59	1 9	1066.8	11:12	24:13
			Subcutaneous				0.17			0.18			0.18			0.19			0.19			
12	092		Gavage	209.49	59	11:22	1.7	1075.3	11:02	1.7	1045.9	12:00	1.7	1022.2	11:25	1.8	1051.0	11:11	1.8	1031.7	11:55	24:44
			Subcutaneous				0.17			0.17			0.17			0.18			0.18			
12	093		Gavage	209.49	60	11:10	1.5	1036.1	12:12	1.5	1025.9	11:37	1.6	1062.1	11:22	1.6	1058.0	10:42	1.6	1042.9	9:34	22 52
			Subcutaneous				0.15			0.15			0.16			0.16			0.16			
12	094		Gavage	209.49	60	11:18	1.4	1012.0	12:26	1.5	1082.1	11 51	1.5	1039.1	11:34	1 5	1024 9	10:53	1 5	1022.6	10:26	23:33
			Subcutaneous				0.14			0.15			0.15			0.15			0.15			
12	095		Gavage	209.49	60	11:27	1.6	1063.4	12:39	1.6	1046.5	12:04	1.6	1042.2	11:46	1.7	1078 9	11:04	1.7	1067.5	11:13	24:09
			Subcutaneous				0.16			0.16			0.16			0.17			0.17			
12	096		Gavage	209.49	60	11:35	1.7	1055.8	12:55	1.7	1058.3	12:16	1.8	1076.8	11 59	1.8	1060.7	11:16	1.8	1065.2	11:56	24:40
			Subcutaneous				0.17			0.17			0.18			0.18			0.18			

Dose Administration:

					Day 1 (6 & 7 April 2012)		Day 2 (7 & 8 April 2012)		Day 3 (8 & 9 April 2012)		Day 4 (9 & 10 April 2012)		Day 5 (10 & 11 April 2012)	
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 Administration (Hour:Minute)	Volume Administered (mL)	Time of Administration (Hour:Minute)	Volume Administered (mL)
13	097	Flutamide (3.0 mg/kg) + TP (0.4 mg/kg)	Gavage	59	10:49	1.5	10:41	1.5	10:44	1.5	10:50	1.5	10:56	1.6
			Subcutaneous			0.15		0.15		0.15		0.15		0.16
13	098		Gavage	59	11:02	1.3	10:57	1.4	10:54	1.4	11:03	1.5	11:05	1.5
			Subcutaneous			0.13		0.14		0.14		0.15		0.15
13	099		Gavage	59	11:15	1.4	11:14	1.4	11:04	1.5	11:14	1.5	11:13	1.5
			Subcutaneous			0.14		0.14		0.15		0.15		0.15
13	100		Gavage	59	11:28	1.4	11:31	1.4	11:13	1.4	11:26	1.5	11:21	1.5
			Subcutaneous			0.14		0.14		0.14		0.15		0.15
13	101		Gavage	60	11:49	1.4	11:25	1.4	11:39	1.5	11:29	1.5	11:42	1.5
			Subcutaneous			0.14		0.14		0.15		0.15		0.15
13	102		Gavage	60	12:03	1.4	11:34	1.4	11:53	1.4	11:41	1.5	11:58	1.5
			Subcutaneous			0.14		0.14		0.14		0.15		0.15
13	103		Gavage	60	12:18	1.5	11:43	1.6	12:06	1.6	11:51	1.7	12:12	1.7
			Subcutaneous			0.15		0.16		0.16		0.17		0.17
13	104		Gavage	60	12:31	1.5	11:53	1.6	12:21	1.6	11:59	1.6	12:27	1.7
			Subcutaneous			0.15		0.16		0.16		0.16		0.17

					Day 6 (11 & 12 April 2012)		Day 7 (12 & 13 April 2012)		Day 8 (13 & 14 April 2012)		Day 9 (14 & 15 April 2012)		Day 10 (15 & 16 April 2012)		Day 11 (16 & 17 April 2012)	
Group No.:	Animal No.:	Test Substance Dose Level	Dose Route	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 Administration (Hour:Minute)	Volume Administered (mL)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Time of Death	Time From Last Administration
13	097	0.00	Gavage	59	10:39	1.6	10:38	1.6	11:11	1.7	10:45	1.7	10:39	1.7	9:36	22:57
			Subcutaneous			0.16		0.16		0.17		0.17		0.17		
13	098		Gavage	59	10:53	1.5	10:45	1.5	11:27	1.6	10:59	1.6	10:49	1.7	10:33	23:44
			Subcutaneous			0.15		0.15		0.16		0.16		0.17		
13	099		Gavage	59	11:10	1.5	10:53	1.6	11:44	1.6	11:12	1.6	11:00	1.6	11:16	24:16
			Subcutaneous			0.15		0.16		0.16		0.16		0.16		
13	100		Gavage	59	11:24	1.5	11:03	1.5	12:01	1.5	11:26	1.6	11:12	1.6	11:58	24:46
			Subcutaneous			0.15		0.15		0.15		0.16		0.16		
13	101		Gavage	60	11:11	1.6	12:14	1.5	11:38	1.6	11:24	1.6	10:43	1.7	9:37	22:54
			Subcutaneous			0.16		0.15		0.16		0.16		0.17		
13	102		Gavage	60	11:19	1.5	12:26	1.5	11:52	1.6	11:36	1.6	10:54	1.6	10:29	23:35
			Subcutaneous			0.15		0.15		0.16		0.16		0.16		
13	103		Gavage	60	11:27	1.8	12:40	1.8	12:05	1.9	11:47	1.9	11:05	1.9	11:16	24:11
			Subcutaneous			0.18		0.18		0.19		0.19		0.19		
13	104		Gavage	60	11:36	1.7	12:56	1.7	12:18	1.8	12:00	1.8	11:17	1.8	11:59	24:42
			Subcutaneous			0.17		0.17		0.18		0.18		0.18		

Appendix IV:

Clinical Observation Data

Clinical Observations

				Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Final
Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**
1	001	M	Corn Oil Control	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1	002	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1	003	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1	004	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1	005	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1	006	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1	007	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
1	008	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**
2	009	M	Ensulizole (320 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	010	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	011	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	012	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	013	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	014	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	015	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
2	016	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**	**Clinical Observation**
3	017	M	Ensulizole (1000 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3	018	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3	019	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3	020	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3	021	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3	022	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3	023	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
3	024	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Clinical Observations

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
4	025	M	Avobenzone (320 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
4	026	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
4	027	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
4	028	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
4	029	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
4	030	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
4	031	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
4	032	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
5	033	M	Avobenzone (1000 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5	034	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5	035	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5	036	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5	037	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5	038	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5	039	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
5	040	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
6	041	M	Corn Oil Control (0 mg/kg) + TP (0.4 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
6	042	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
6	043	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
6	044	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
6	045	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
6	046	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
6	047	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
6	048	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Clinical Observations

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
7	049	M	Ensulizole (100 mg/kg) + TP (0.4 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	050	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	051	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	052	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	053	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	054	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	055	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	056	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
8	057	M	Ensulizole (320 mg/kg) + TP (0.4 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	058	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	059	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	060	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	061	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	062	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	063	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	064	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
9	065	M	Ensulizole (1000 mg/kg) + TP (0.4 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	066	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	067	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	068	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	069	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	070	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	071	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
9	072	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Clinical Observations

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
10	073	M	Avobenzone (100 mg/kg) + TP (0.4 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
10	074	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
10	075	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
10	076	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
10	077	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
10	078	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
10	079	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
10	080	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
11	081	M	Avobenzone (320 mg/kg) + TP (0.4 mg/kg)	Normal	Normal	Normal	Normal	Normal	Nomal	Normal	Normal	Normal	Normal	Normal
11	082	M		Normal	Normal	Normal	Normal	Normal	Nomal	Normal	Normal	Normal	Normal	Normal
11	083	M		Normal	Normal	Normal	Normal	Normal	Nomal	Normal	Normal	Normal	Normal	Normal
11	084	M		Normal	Normal	Normal	Normal	Normal	Nomal	Normal	Normal	Normal	Normal	Normal
11	085	M		Normal	Normal	Normal	Normal	Normal	Nomal	Normal	Normal	Normal	Normal	Normal
11	086	M		Normal	Normal	Normal	Normal	Normal	Nomal	Normal	Normal	Normal	Normal	Normal
11	087	M		Normal	Normal	Normal	Normal	Normal	Nomal	Normal	Normal	Normal	Normal	Normal
11	088	M		Normal	Normal	Normal	Normal	Normal	Nomal	Normal	Normal	Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
12	089	M	Avobenzone (1000 mg/kg) + TP (0.4 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	090	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	091	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	092	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	093	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	094	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	095	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
12	096	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation	Clinical Observation
13	097	M	Flutamide (3.0 mg/kg) + TP (0.4 mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
13	098	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Scab(near left eye)	Scab(near left and right eye)
13	099	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
13	100	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
13	101	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
13	102	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
13	103	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal
13	104	M		Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal	Normal

Appendix V:

Body Weight Data

Body Weights

				Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7	Day 8	Day 9	Day 10	Final	
Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight Gain (g)**
1	001	M	Corn Oil Control	316.6	317.4	325.8	333.1	337.2	342.3	342.4	342.3	358.2	359.2	360.0	43.4
1	002	M		249.7	255.0	261.0	267.3	269.8	275.1	277.2	275.0	285.1	288.5	281.9	32.2
1	003	M		275.2	278.3	286.7	295.3	294.5	304.1	306.1	300.1	309.8	315.7	315.2	40.0
1	004	M		287.9	288.2	297.5	305.7	305.5	314.4	320.3	316.3	328.1	333.9	334.5	46.6
1	005	M		297.5	303.8	313.2	312.1	316.9	327.5	323.9	330.4	337.5	337.7	342.7	45.2
1	006	M		300.4	304.0	319.7	313.1	320.8	328.3	324.9	338.1	343.7	348.5	346.7	46.3
1	007	M		286.5	294.2	297.8	299.9	304.3	306.4	306.6	315.6	317.2	321.9	320.0	33.5
1	008	M		273.3	275.5	278.2	281.4	287.2	286.6	286.9	293.5	298.3	298.9	299.0	25.7
			Mean	**285.9**	**289.6**	**297.5**	**301.0**	**304.5**	**310.6**	**311.0**	**313.9**	**322.2**	**325.5**	**325.0**	**39.1**
			SD	**20.3**	**19.7**	**21.9**	**20.3**	**21.0**	**22.4**	**21.4**	**23.3**	**24.4**	**24.1**	**26.1**	**7.8**

Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight Gain (g)**
2	009	M	Ensulizole (320 mg/kg)	293.0	297.3	302.5	309.6	312.2	315.0	323.4	323.2	331.2	333.7	335.3	42.3
2	010	M		285.5	287.9	294.5	302.2	308.5	312.4	314.8	317.0	324.3	323.4	325.1	39.6
2	011	M		283.9	285.5	292.8	301.1	304.2	311.5	313.5	313.3	324.2	326.2	334.8	50.9
2	012	M		255.2	259.4	264.5	269.0	269.8	272.5	278.5	278.9	280.8	281.3	385.7	130.5
2	013	M		294.0	297.2	304.5	313.4	316.5	321.9	320.6	329.7	332.8	336.7	340.7	46.7
2	014	M		281.5	283.0	282.6	288.4	292.2	297.2	295.9	304.4	308.8	312.6	315.5	34.0
2	015	M		330.6	330.6	342.1	347.1	354.8	355.3	358.6	368.9	372.9	379.0	380.5	49.9
2	016	M		274.5	282.0	290.6	296.2	298.8	304.9	306.1	316.1	323.0	320.0	324.6	50.1
			Mean	**287.3**	**290.4**	**296.8**	**303.4**	**307.1**	**311.3**	**313.9**	**318.9**	**324.8**	**326.6**	**342.8**	**55.5**
			SD	**21.4**	**20.1**	**22.2**	**22.4**	**24.1**	**23.3**	**23.2**	**25.3**	**25.7**	**27.3**	**26.1**	**30.9**
			% of Control	**100.5**	**100.3**	**99.8**	**100.8**	**100.9**	**100.2**	**100.9**	**101.6**	**100.8**	**100.3**	**105.5**	

Group No.:	**Animal No.:**	**Sex**	**Test Substance Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight Gain (g)**
3	017	M	Ensulizole (1000 mg/kg)	279.4	280.5	287.7	293.7	292.0	300.3	302.9	303.6	312.0	315.7	315.3	35.9
3	018	M		320.2	324.9	331.1	337.4	344.7	353.3	354.8	358.1	369.5	373.1	377.4	57.2
3	019	M		280.7	276.6	281.3	287.6	290.0	294.0	296.5	294.8	304.1	305.6	305.1	24.4
3	020	M		273.2	272.6	277.7	285.7	287.6	289.4	293.5	295.2	301.7	304.5	307.9	34.7
3	021	M		257.6	260.3	262.7	265.6	268.6	272.7	269.4	277.1	279.4	281.8	279.3	21.7
3	022	M		302.9	304.9	313.3	315.3	318.9	324.2	322.7	331.1	335.7	337.9	341.2	38.3
3	023	M		280.8	281.2	288.4	289.5	292.5	297.2	297.9	301.4	303.4	304.4	305.6	24.8
3	024	M		307.2	305.8	317.5	319.2	320.5	324.7	331.2	336.1	340.4	341.3	347.2	40.0
			Mean	**287.8**	**288.4**	**295.0**	**299.3**	**301.9**	**307.0**	**308.6**	**312.2**	**318.3**	**320.5**	**322.4**	**34.6**
			SD	**20.5**	**21.4**	**23.2**	**23.0**	**24.2**	**25.5**	**26.5**	**26.9**	**28.4**	**28.7**	**30.9**	**11.5**
			% of Control	**100.7**	**99.6**	**99.2**	**99.4**	**99.1**	**98.8**	**99.2**	**99.4**	**98.8**	**98.5**	**99.2**	

Body Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight Gain (g)
4	025	M	Avobenzone (320 mg/kg)	257.9	259.9	264.5	273.5	277.2	284.2	290.1	282.5	300.7	299.9	304.4	46.5
4	026	M		268.4	274.6	277.0	284.4	289.2	291.3	290.2	285.4	294.5	301.2	295.0	26.6
4	027	M		315.9	317.2	318.8	329.4	335.3	340.7	344.5	347.4	356.1	360.5	361.0	45.1
4	028	M		296.7	300.8	300.0	308.8	309.8	309.5	314.6	315.0	317.0	317.9	317.5	20.8
4	029	M		283.9	286.0	295.3	292.0	297.6	297.1	299.2	300.1	302.6	304.6	305.9	22.0
4	030	M		290.3	294.2	301.4	299.7	304.1	308.0	302.6	310.2	314.8	319.9	317.0	26.7
4	031	M		275.5	280.8	286.1	289.8	289.6	297.5	299.6	300.5	305.7	308.6	314.4	38.9
4	032	M		296.0	298.2	304.8	304.1	308.1	316.9	316.4	321.8	320.7	322.9	326.4	30.4
			Mean	**285.6**	**289.0**	**293.5**	**297.7**	**301.4**	**305.7**	**307.2**	**307.9**	**314.0**	**316.9**	**317.7**	**32.1**
			SD	**18.3**	**17.6**	**17.1**	**17.0**	**17.6**	**17.7**	**17.9**	**21.0**	**19.2**	**19.6**	**20.0**	**10.1**
			% of Control	**99.9**	**99.8**	**98.7**	**98.9**	**99.0**	**98.4**	**98.8**	**98.1**	**97.4**	**97.4**	**97.8**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight Gain (g)
5	033	M	Avobenzone (1000 mg/kg)	317.6	311.7	314.7	319.4	325.3	332.1	339.7	337.9	346.3	351.3	351.7	34.1
5	034	M		276.2	276.6	280.4	281.9	287.2	289.4	289.9	290.6	298.1	302.1	302.9	26.7
5	035	M		280.6	280.4	285.0	289.4	294.0	297.7	305.4	299.7	304.5	307.3	312.6	32.0
5	036	M		281.5	281.5	284.4	290.2	295.3	299.2	303.7	302.5	310.8	313.6	318.5	37.0
5	037	M		310.4	310.2	320.4	328.8	335.3	334.6	338.6	345.1	349.7	350.1	352.4	42.0
5	038	M		299.9	302.9	308.9	317.0	318.2	315.6	318.7	331.8	337.1	339.0	338.7	38.8
5	039	M		273.2	269.5	284.7	286.5	286.1	285.2	289.7	294.1	295.8	300.7	306.8	33.6
5	040	M		262.4	261.5	264.1	264.9	268.9	267.4	267.4	272.1	275.7	271.3	273.1	10.7
			Mean	**287.7**	**286.8**	**292.8**	**297.3**	**301.3**	**302.7**	**306.6**	**309.2**	**314.8**	**316.9**	**319.6**	**31.9**
			SD	**19.4**	**19.0**	**19.5**	**22.0**	**22.6**	**23.3**	**25.0**	**25.9**	**26.7**	**27.9**	**27.1**	**9.7**
			% of Control	**100.6**	**99.0**	**98.4**	**98.8**	**98.9**	**97.4**	**98.6**	**98.5**	**97.7**	**97.4**	**98.3**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight Gain (g)
6	041	M	Corn Oil Control (0 mg/kg) + TP (0.4 mg/kg)	305.2	305.9	315.5	329.2	332.3	344.3	347.8	350.2	357.5	361.8	365.4	60.2
6	042	M		256.6	257.8	265.6	273.3	287.7	295.1	298.1	300.9	307.8	313.4	314.6	58.0
6	043	M		273.0	276.1	268.8	292.6	296.1	303.3	312.9	312.6	324.9	329.1	334.5	61.5
6	044	M		296.6	303.6	291.0	318.3	322.4	333.5	342.6	349.0	361.9	368.4	373.1	76.5
6	045	M		297.1	297.7	309.2	313.6	327.7	336.6	337.4	345.4	350.3	356.3	356.5	59.4
6	046	M		296.1	298.3	307.5	316.9	327.1	335.4	341.6	349.0	357.7	365.4	367.1	71.0
6	047	M		275.5	281.9	294.7	306.1	317.7	326.6	335.2	346.2	355.7	359.2	360.9	85.4
6	048	M		288.7	297.2	304.5	313.5	321.2	328.3	329.1	339.6	342.3	349.0	349.5	60.8
			Mean	**286.1**	**289.8**	**294.6**	**307.9**	**316.5**	**325.4**	**330.6**	**336.6**	**344.8**	**350.3**	**352.7**	**66.6**
			SD	**16.3**	**16.5**	**18.6**	**17.5**	**16.0**	**17.2**	**16.9**	**19.0**	**19.1**	**19.3**	**19.5**	**10.0**

Body Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight Gain (g)
7	049	M	Ensulizole (100 mg/kg) + TP (0.4 mg/kg)	266.8	272.8	277.2	288.7	294.5	303.6	310.6	312.0	326.2	331.3	333.7	66.9
7	050	M		308.1	307.5	313.1	325.0	335.5	341.8	353.4	344.6	357.5	366.1	366.1	58.0
7	051	M		265.5	265.5	277.1	288.3	297.2	306.7	317.4	313.0	327.2	342.3	343.1	77.6
7	052	M		297.6	304.6	312.0	319.8	329.8	338.5	349.6	350.3	360.7	370.2	374.3	76.7
7	053	M		299.4	302.0	314.3	325.0	335.6	345.2	352.5	364.0	372.5	373.0	379.2	79.8
7	054	M		290.3	295.3	303.6	310.2	323.9	327.0	332.5	344.9	351.6	361.2	363.3	73.0
7	055	M		297.7	297.5	310.4	313.4	323.7	330.6	334.7	344.7	347.6	353.1	356.4	58.7
7	056	M		277.9	279.4	288.3	291.5	302.1	304.6	309.5	314.6	320.9	325.6	329.5	51.6
			Mean	**287.9**	**290.6**	**299.5**	**307.7**	**317.8**	**324.8**	**332.5**	**336.0**	**345.5**	**352.9**	**355.7**	**67.8**
			SD	**16.0**	**15.8**	**16.1**	**16.0**	**17.2**	**17.4**	**18.4**	**19.9**	**18.7**	**18.0**	**18.5**	**10.6**
			% of Control	**100.6**	**100.3**	**101.7**	**99.9**	**100.4**	**99.8**	**100.6**	**99.8**	**100.2**	**100.7**	**100.9**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight Gain (g)
8	057	M	Ensulizole (320 mg/kg) + TP (0.4 mg/kg)	256.5	261.3	271.7	278.0	288.6	300.0	308.7	316.7	326.6	333.7	337.9	81.4
8	058	M		310.9	314.2	324.8	340.6	349.4	361.0	369.0	375.6	395.4	400.9	407.1	96.2
8	059	M		289.7	295.3	302.4	310.9	321.6	331.2	338.3	337.2	357.1	365.9	368.3	78.6
8	060	M		279.3	286.6	294.3	297.8	313.7	319.2	330.2	335.4	346.5	354.6	355.9	76.6
8	061	M		291.5	300.2	302.8	312.9	323.7	326.4	333.0	341.8	347.8	356.2	357.1	65.6
8	062	M		299.3	304.8	320.1	324.9	331.0	336.3	346.1	355.2	362.7	369.2	372.3	73.0
8	063	M		286.1	295.4	306.4	311.7	316.3	322.3	326.8	333.7	340.8	345.2	345.6	59.5
8	064	M		272.4	281.8	295.8	304.6	319.1	328.8	335.1	345.6	355.6	364.0	371.9	99.5
			Mean	**285.7**	**292.5**	**302.3**	**310.2**	**320.4**	**328.2**	**335.9**	**342.7**	**354.1**	**361.2**	**364.5**	**78.8**
			SD	**16.6**	**16.1**	**16.4**	**18.4**	**17.1**	**17.2**	**17.2**	**17.3**	**20.1**	**19.8**	**21.2**	**13.7**
			% of Control	**99.9**	**100.9**	**102.6**	**100.7**	**101.2**	**100.8**	**101.6**	**101.8**	**102.7**	**103.1**	**103.3**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight Gain (g)
9	065	M	Ensulizole (1000 mg/kg) + TP (0.4 mg/kg)	297.3	301.1	305.3	319.6	329.9	337.7	348.3	353.5	363.2	374.4	378.1	80.8
9	066	M		298.8	302.9	307.2	318.9	325.2	334.4	344.6	348.1	355.5	364.2	369.9	71.1
9	067	M		276.7	280.4	288.6	298.9	304.1	310.4	312.9	313.1	323.9	326.1	331.9	55.2
9	68#	M		269.3	274.8	282.7	293.4	301.6	310.9	322.2	323.9	335.2	342.7		
9	069	M		281.5	288.6	303.8	317.1	329.5	336.9	344.5	360.3	367.4	377.7	385.1	103.6
9	070	M		268.3	274.7	287.6	296.6	304.3	309.9	312.4	322.6	330.4	336.6	338.3	70.0
9	071	M		304.7	306.1	314.7	318.1	330.1	339.5	339.5	351.9	360.9	366.4	368.4	63.7
9	072	M		293.3	295.7	307.1	310.7	316.1	321.4	315.6	331.5	334.1	339.3	340.9	47.6
			Mean	**286.2**	**290.5**	**299.6**	**309.2**	**317.6**	**325.1**	**330.0**	**338.1**	**346.3**	**353.4**	**358.9**	**70.3**
			SD	**14.1**	**12.8**	**11.6**	**11.1**	**12.7**	**13.4**	**15.7**	**17.4**	**17.1**	**19.5**	**21.4**	**18.3**
			% of Control	**100.0**	**100.3**	**101.7**	**100.4**	**100.3**	**99.9**	**99.8**	**100.4**	**100.5**	**100.9**	**101.8**	

#Final body weight and body weight gain removed due to possible recording error and excluded from statistical analysis

Body Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight Gain (g)
10	073	M	Avobenzone (100 mg/kg) + TP (0.4 mg/kg)	278.4	282.9	290.0	298.1	308.7	316.7	321.7	330.5	336.8	341.7	349.3	70.9
10	074	M		304.8	308.8	317.3	331.3	335.5	344.9	345.5	356.3	369.4	372.3	376.3	71.5
10	075	M		274.5	279.3	286.7	297.6	305.6	313.0	317.0	321.6	333.1	333.5	337.4	62.9
10	076	M		271.7	276.2	285.0	298.9	303.7	315.7	323.5	331.7	338.0	343.6	339.5	67.8
10	077	M		294.7	300.0	312.5	320.4	330.4	336.5	340.8	348.2	357.4	367.3	373.0	78.3
10	078	M		314.1	324.1	333.5	344.3	365.2	363.8	370.9	385.1	392.7	400.3	407.4	93.3
10	079	M		273.9	280.0	291.7	298.5	310.1	314.3	319.0	329.2	328.2	334.9	339.7	65.8
10	080	M		292.8	298.5	308.4	319.4	326.6	336.4	340.4	344.5	345.2	357.9	359.6	66.8
			Mean	**288.1**	**293.7**	**303.1**	**313.6**	**323.2**	**330.2**	**334.9**	**343.4**	**350.1**	**356.4**	**360.3**	**72.2**
			SD	**15.9**	**17.0**	**17.5**	**18.0**	**20.9**	**18.4**	**18.3**	**20.4**	**21.9**	**22.9**	**24.3**	**9.7**
			% of Control	**100.7**	**101.4**	**102.9**	**101.8**	**102.1**	**101.5**	**101.3**	**102.0**	**101.5**	**101.7**	**102.1**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight Gain (g)
11	081	M	Avobenzone (320 mg/kg) + TP (0.4 mg/kg)	292.3	292.6	299.7	315.2	320.2	328.9	339.4	341.8	352.6	358.6	359.1	66.8
11	082	M		308.5	309.2	314.5	331.2	334.4	343.7	353.2	354.6	366.7	376.4	384.1	75.6
11	083	M		275.2	281.0	289.0	297.2	306.2	316.1	325.1	321.8	338.0	341.4	343.8	68.6
11	084	M		273.2	276.3	284.1	289.8	296.7	303.2	311.5	312.2	324.3	330.5	336.4	63.2
11	085	M		273.1	275.3	290.6	297.5	305.5	304.3	307.5	318.5	323.0	329.0	330.2	57.1
11	086	M		295.7	300.8	310.7	323.1	334.7	339.8	344.2	359.0	358.3	366.7	370.0	74.3
11	087	M		302.8	310.8	323.8	329.5	339.1	346.8	347.7	358.6	364.9	367.7	374.9	72.1
11	088	M		268.5	272.5	283.2	291.1	296.7	300.6	302.9	312.1	319.2	320.4	323.9	55.4
			Mean	**286.2**	**289.8**	**299.5**	**309.3**	**316.7**	**322.9**	**328.9**	**334.8**	**343.4**	**348.8**	**352.8**	**66.6**
			SD	**15.5**	**15.7**	**15.3**	**17.4**	**17.7**	**19.3**	**19.8**	**20.9**	**19.7**	**21.1**	**22.4**	**7.6**
			% of Control	**100.0**	**100.0**	**101.6**	**100.5**	**100.1**	**99.2**	**99.5**	**99.5**	**99.6**	**99.6**	**100.0**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight Gain (g)
12	089	M	Avobenzone (1000 mg/kg) + TP (0.4 mg/kg)	272.8	275.2	278.1	291.5	300.5	304.7	313.2	311.6	318.9	325.3	330.3	57.5
12	090	M		269.7	268.2	268.1	278.9	283.4	288.7	293.3	286.6	297.1	305.4	310.0	40.3
12	091	M		308.3	306.2	319.3	328.5	335.3	345.7	352.2	359.2	372.1	373.1	380.3	72.0
12	092	M		293.0	294.8	305.7	315.0	326.9	331.2	340.5	348.4	358.8	365.5	374.5	81.5
12	093	M		280.1	277.7	287.6	275.0	293.0	303.3	306.3	315.6	316.8	321.4	327.9	47.8
12	094	M		266.3	270.4	274.8	263.6	282.2	289.8	290.4	302.4	306.6	307.3	311.6	45.3
12	095	M		310.2	309.2	313.4	314.4	322.1	315.2	320.3	321.6	330.1	333.6	341.1	30.9
12	096	M		294.7	303.5	308.1	312.6	325.7	337.3	336.5	350.2	355.5	354.0	358.0	63.3
			Mean	**286.9**	**288.2**	**294.4**	**297.4**	**308.6**	**314.5**	**319.1**	**324.5**	**332.0**	**335.7**	**341.7**	**54.8**
			SD	**17.2**	**17.1**	**19.6**	**23.4**	**21.3**	**21.6**	**22.5**	**25.7**	**27.1**	**25.8**	**26.9**	**16.9**
			% of Control	**100.3**	**99.4**	**99.9**	**96.6**	**97.5**	**96.7**	**96.5**	**96.4**	**96.3**	**95.8**	**96.9**	

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight Gain (g)
13	097	M	Flutamide (3.0 mg/kg) + TP (0.4 mg/kg)	290.3	291.7	300.2	308.1	316.6	325.1	326.0	331.9	343.5	348.6	351.2	60.9
13	098	M		268.8	271.5	278.9	291.2	298.7	306.4	308.4	314.0	327.3	333.1	338.3	69.5
13	099	M		287.3	285.9	293.3	299.2	303.6	308.5	312.5	315.7	324.4	329.2	333.2	45.9
13	100	M		274.9	279.1	288.8	293.2	292.3	299.5	302.4	307.0	310.7	314.6	316.9	42.0
13	101	M		277.9	285.2	292.4	301.7	303.8	310.3	309.5	321.7	324.3	331.4	331.8	53.9
13	102	M		270.7	274.5	285.9	291.3	296.3	306.5	307.9	319.2	320.2	326.0	329.2	58.5
13	103	M		307.3	314.4	328.4	335.4	344.7	357.2	354.2	371.1	380.4	381.9	385.4	78.1
13	104	M		303.2	310.0	318.2	328.2	335.4	345.5	340.9	360.5	365.5	368.1	370.4	67.2
			Mean	**285.1**	**289.0**	**298.3**	**306.0**	**311.4**	**319.9**	**320.2**	**330.1**	**337.0**	**341.6**	**344.6**	**59.5**
			SD	**14.5**	**15.7**	**16.8**	**17.0**	**19.2**	**21.0**	**18.5**	**23.3**	**24.3**	**22.9**	**23.0**	**12.1**
			% of Control	**99.6**	**99.7**	**101.2**	**99.4**	**98.4**	**98.3**	**96.9**	**98.1**	**97.8**	**97.5**	**97.7**	

Appendix VI:

Tissue Weight Data

Tissue Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
1	001	M	Corn Oil Control	360.0	0.0629	0.0311	0.0670	0.0118	0.2143
1	002	M		281.9	0.0595	0.0387	0.0575	0.0095	0.1051
1	003	M		315.2	0.0626	0.0192	0.0666	0.0035	0.1611
1	004	M		334.5	0.0574	0.0205	0.0588	0.0074	0.0982
1	005	M		342.7	0.0487	0.0219	0.0648	0.0116	0.1150
1	006	M		346.7	0.0627	0.0264	0.0615	0.0063	0.1120
1	007	M		320.0	0.0659	0.0210	0.0563	0.0065	0.1494
1	008	M		299.0	0.0535	0.0239	0.0670	0.0061	0.1164
			Mean	**325.0**	**0.0592**	**0.0253**	**0.0624**	**0.0078**	**0.1339**
			SD	**26.1**	**0.0057**	**0.0066**	**0.0045**	**0.0029**	**0.0391**
			CV	**8.0**	**9.7**	**26.1**	**7.2**	**37.0**	**29.2**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
2	009	M	Ensulizole (320 mg/kg)	335.3	0.0662	0.0218	0.0674	0.0052	0.1714
2	010	M		325.1	0.0627	0.0247	0.0661	0.0071	0.1438
2	011	M		334.8	0.0642	0.0227	0.0762	0.0054	0.1494
2	012	M		385.7	0.0598	0.0126	0.0496	0.0074	0.1291
2	013	M		340.7	0.0620	0.0188	0.0463	0.0132	0.1336
2	014	M		315.5	0.0587	0.0150	0.0501	0.0049	0.1359
2	015	M		380.5	0.0515	0.0183	0.0690	0.0072	0.1444
2	016	M		324.6	0.0632	0.0187	0.0572	0.0090	0.1550
			Mean	**342.8**	**0.0610**	**0.0191**	**0.0602**	**0.0074**	**0.1453**
			SD	**26.1**	**0.0045**	**0.0040**	**0.0109**	**0.0027**	**0.0135**
			CV	**7.6**	**7.4**	**20.9**	**18.1**	**36.5**	**9.3**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
3	017	M	Ensulizole (1000 mg/kg)	315.3	0.0660	0.0191	0.0678	0.0056	0.1364
3	018	M		377.4	0.0745	0.0184	0.0774	0.0091	0.1913
3	019	M		305.1	0.0622	0.0143	0.0618	0.0095	0.1515
3	020	M		307.9	0.0564	0.0350	0.0550	0.0084	0.1290
3	021	M		279.3	0.0550	0.0206	0.0532	0.0068	0.1175
3	022	M		341.2	0.0578	0.0193	0.0621	0.0056	0.1220
3	023	M		305.6	0.0657	0.0250	0.0834	0.0059	0.1473
3	024	M		347.2	0.0684	0.0207	0.0722	0.0090	0.1295
			Mean	**322.4**	**0.0633**	**0.0216**	**0.0666**	**0.0075**	**0.1406**
			SD	**30.9**	**0.0067**	**0.0062**	**0.0106**	**0.0017**	**0.0236**
			CV	**9.6**	**10.6**	**28.7**	**15.9**	**22.5**	**16.8**

Tissue Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
4	025	M	Avobenzone (320 mg/kg)	304.4	0.0591	0.0166	0.0537	0.0065	0.1387
4	026	M		295.0	0.0560	0.0166	0.0394	0.0062	0.1705
4	027	M		361.0	0.0698	0.0179	0.0701	0.0073	0.1528
4	028	M		317.5	0.0626	0.0194	0.0805	0.0111	0.2408
4	029	M		305.9	0.0567	0.0172	0.0548	0.0043	0.0870
4	030	M		317.0	0.0593	0.0221	0.0576	0.0071	0.1572
4	031	M		314.4	0.0618	0.0227	0.0484	0.0068	0.1274
4	032	M		326.4	0.0602	0.0221	0.0621	0.0132	0.1180
			Mean	**317.7**	**0.0607**	**0.0193**	**0.0583**	**0.0078**	**0.1491**
			SD	**20.0**	**0.0043**	**0.0026**	**0.0127**	**0.0029**	**0.0453**
			CV	**6.3**	**7.1**	**13.6**	**21.8**	**36.9**	**30.4**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
5	033	M	Avobenzone (1000 mg/kg)	351.7	0.0649	0.0239	0.0631	0.0077	0.1990
5	034	M		302.9	0.0569	0.0132	0.0492	0.0081	0.1148
5	035	M		312.6	0.0651	0.0248	0.0621	0.0068	0.1308
5	036	M		318.5	0.0582	0.0170	0.0627	0.0061	0.1325
5	037	M		352.4	0.0583	0.0192	0.0618	0.0059	0.1427
5	038	M		338.7	0.0528	0.0238	0.0543	0.0047	0.1337
5	039	M		306.8	0.0565	0.0158	0.0562	0.0039	0.1140
5	040	M		273.1	0.0662	0.0274	0.0729	0.0070	0.1467
			Mean	**319.6**	**0.0599**	**0.0206**	**0.0603**	**0.0063**	**0.1393**
			SD	**27.1**	**0.0049**	**0.0050**	**0.0071**	**0.0014**	**0.0268**
			CV	**8.5**	**8.2**	**24.4**	**11.8**	**22.9**	**19.2**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
6	041	M	Corn Oil Control (0 mg/kg) + TP (0.4 mg/kg)	365.4	0.1105	0.1516	0.9070	0.0448	0.4590
6	042	M		314.6	0.1008	0.1212	0.9124	0.0544	0.3743
6	043	M		334.5	0.0966	0.2098	0.6507	0.0451	0.4774
6	044	M		373.1	0.1014	0.2137	0.8760	0.0720	0.4928
6	045	M		356.5	0.0881	0.1798	0.7157	0.0306	0.4079
6	046	M		367.1	0.0927	0.1849	0.6690	0.0397	0.3597
6	047	M		360.9	0.1015	0.1871	0.5248	0.0377	0.4090
6	048	M		349.5	0.1071	0.1630	0.8652	0.0344	0.5442
			Mean	**352.7**	**0.0998**	**0.1764**	**0.7651**	**0.0448**	**0.4405**
			SD	**19.5**	**0.0073**	**0.0306**	**0.1447**	**0.0132**	**0.0634**
			CV	**5.5**	**7.3**	**17.3**	**18.9**	**29.4**	**14.4**

Tissue Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
7	049	M	Ensulizole (100 mg/kg) + TP (0.4 mg/kg)	333.7	0.0995	0.2148	0.8395	0.0480	0.3721
7	050	M		366.1	0.1065	0.2763	0.7890	0.0309	0.4308
7	051	M		343.1	0.0947	0.1680	0.7569	0.0449	0.4394
7	052	M		374.3	0.1071	0.2472	0.8895	0.0513	0.4132
7	053	M		379.2	0.1008	0.2614	0.9162	0.0568	0.4679
7	054	M		363.3	0.0922	0.2270	0.8886	0.0530	0.5132
7	055	M		356.4	0.0944	0.2111	0.5301	0.0343	0.3438
7	056	M		329.5	0.0996	0.2257	0.8309	0.0566	0.4090
			Mean	**355.7**	**0.0994**	**0.2289**	**0.8051**	**0.0470**	**0.4237**
			SD	**18.5**	**0.0055**	**0.0335**	**0.1233**	**0.0098**	**0.0530**
			CV	**5.2**	**5.5**	**14.7**	**15.3**	**20.8**	**12.5**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
8	057	M	Ensulizole (320 mg/kg) + TP (0.4 mg/kg)	337.9	0.0956	0.2192	0.7091	0.0434	0.4374
8	058	M		407.1	0.1076	0.2401	0.8057	0.0526	0.4932
8	059	M		368.3	0.1012	0.2058	0.8758	0.0667	0.4325
8	060	M		355.9	0.0930	0.1992	0.8836	0.0553	0.4190
8	061	M		357.1	0.1007	0.1917	0.8790	0.0523	0.4699
8	062	M		372.3	0.1037	0.2664	1.0561	0.0680	0.4820
8	063	M		345.6	0.0959	0.1944	0.8177	0.0445	0.3396
8	064	M		371.9	0.0960	0.2177	0.8492	0.0493	0.4790
			Mean	**364.5**	**0.0992**	**0.2168**	**0.8595**	**0.0540**	**0.4441**
			SD	**21.2**	**0.0049**	**0.0256**	**0.0980**	**0.0092**	**0.0499**
			CV	**5.8**	**5.0**	**11.8**	**11.4**	**17.0**	**11.2**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
9	065	M	Ensulizole (1000 mg/kg) + TP (0.4 mg/kg)	378.1	0.1046	0.3910	0.9842	0.0611	0.4418
9	066	M		369.9	0.1124	0.3013	0.9354	0.0506	0.5689
9	067	M		331.9	0.1038	0.2100	0.8537	0.0459	0.4667
9	68#	M			0.0941	0.2661	0.8306	0.0557	0.4485
9	069	M		385.1	0.1010	0.2362	1.0147	0.0516	0.5252
9	070	M		338.3	0.0952	0.1602	0.7219	0.0555	0.3596
9	071	M		368.4	0.1142	0.2040	0.8059	0.0510	0.4938
9	072	M		340.9	0.0982	0.2038	0.9259	0.0351	0.4112
			Mean	**358.9**	**0.1029**	**0.2466**	**0.8840**	**0.0508**	**0.4645**
			SD	**21.4**	**0.0074**	**0.0725**	**0.0983**	**0.0078**	**0.0655**
			CV	**6.0**	**7.2**	**29.4**	**11.1**	**15.3**	**14.1**

#Final body weight removed due to possible recording error and excluded from statistical analysis

Tissue Weights

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
10	073	M	Avobenzone (100 mg/kg) + TP (0.4 mg/kg)	349.3	0.0988	0.1612	0.8289	0.0560	0.4365
10	074	M		376.3	0.1045	0.1955	0.8471	0.0523	0.4930
10	075	M		337.4	0.1040	0.2398	1.0953	0.0628	0.3972
10	076	M		339.5	0.0998	0.1885	0.8244	0.0489	0.4157
10	077	M		373.0	0.1086	0.2031	0.6293	0.0522	0.4354
10	078	M		407.4	0.0949	0.2302	0.7820	0.0378	0.3411
10	079	M		339.7	0.1005	0.2158	0.7619	0.0523	0.3540
10	080	M		359.6	0.1052	0.1977	0.8450	0.0449	0.3517
			Mean	**360.3**	**0.1020**	**0.2040**	**0.8267**	**0.0509**	**0.4031**
			SD	**24.3**	**0.0043**	**0.0248**	**0.1299**	**0.0074**	**0.0526**
			CV	**6.7**	**4.3**	**12.1**	**15.7**	**14.6**	**13.0**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
11	081	M	Avobenzone (320 mg/kg) + TP (0.4 mg/kg)	359.1	0.0983	0.1825	0.7351	0.0519	0.4046
11	082	M		384.1	0.0970	0.2300	0.9018	0.0522	0.3920
11	083	M		343.8	0.0840	0.2311	0.5447	0.0501	0.3654
11	084	M		336.4	0.0939	0.2390	0.8315	0.0505	0.4061
11	085	M		330.2	0.1080	0.2168	0.7829	0.0439	0.3948
11	086	M		370.0	0.0961	0.2187	0.0410#	0.8973#	0.3971
11	087	M		374.9	0.0999	0.2202	0.7788	0.0621	0.4652
11	088	M		323.9	0.0992	0.1946	0.7222	0.0357	0.3538
			Mean	**352.8**	**0.0971**	**0.2166**	**0.7567**	**0.0495**	**0.3974**
			SD	**22.4**	**0.0067**	**0.0191**	**0.1114**	**0.0081**	**0.0331**
			CV	**6.3**	**6.9**	**8.8**	**14.7**	**16.4**	**8.3**

#Seminal Vesicle and Cowper's Gland weights possibly transposed during recording of data and exluded from statistical analysis

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
12	089	M	Avobenzone (1000 mg/kg) + TP (0.4 mg/kg)	330.3	0.1089	0.2223	0.8514	0.0510	0.4611
12	090	M		310.0	0.1027	0.1985	0.7869	0.0564	0.3787
12	091	M		380.3	0.1003	0.1731	0.9127	0.0700	0.3896
12	092	M		374.5	0.1005	0.2148	0.6874	0.0511	0.4493
12	093	M		327.9	0.0964	0.2033	0.6932	0.0552	0.3925
12	094	M		311.6	0.0983	0.2017	0.7754	0.0425	0.4804
12	095	M		341.1	0.1014	0.2094	0.8184	0.0379	0.4461
12	096	M		358.0	0.0941	0.1873	0.6375	0.0391	0.3857
			Mean	**341.7**	**0.1003**	**0.2013**	**0.7704**	**0.0504**	**0.4229**
			SD	**26.9**	**0.0044**	**0.0155**	**0.0925**	**0.0106**	**0.0403**
			CV	**7.9**	**4.4**	**7.7**	**12.0**	**21.1**	**9.5**

Group No.:	Animal No.:	Sex	Test Substance Dose Level	Final Body Weight (g)	Glans Penis Weight (g)	Ventral Prostate Weight (g)	Seminal Vesicle Weight (g)	Cowper's Gland Weight (g)	LABC Weight (g)
13	097	M	Flutamide (3.0 mg/kg) + TP (0.4 mg/kg)	351.2	0.0758	0.0475	0.0893	0.0230	0.2046
13	098	M		338.3	0.0789	0.0420	0.1167	0.0289	0.2333
13	099	M		333.2	0.0630	0.0634	0.2765	0.0220	0.1666
13	100	M		316.9	0.0851	0.0592	0.1524	0.0230	0.2225
13	101	M		331.8	0.0643	0.0405	0.1001	0.0179	0.1864
13	102	M		329.2	0.0604	0.0527	0.1057	0.0166	0.1723
13	103	M		385.4	0.0707	0.0488	0.2051	0.0132	0.2061
13	104	M		370.4	0.0816	0.0492	0.1005	0.0142	0.1749
			Mean	**344.6**	**0.0725**	**0.0504**	**0.1433**	**0.0199**	**0.1958**
			SD	**23.0**	**0.0093**	**0.0079**	**0.0659**	**0.0053**	**0.0246**
			CV	**6.7**	**12.8**	**15.6**	**46.0**	**26.7**	**12.6**

Appendix VII:

Study Protocol

Study Title
The Hershberger Bioassay for Ensulizole and Avobenzone

ILS Project-Study Numbers
N135-248

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

Sponsor
National Toxicology Program
National Institute of Environmental Health Sciences

Study Protocol Approval

[redacted]

3/23/12
Date

Chief, Toxicology Branch
National Toxicology Program, NIEHS

[redacted]

3/26/12
Date

Contract Office Technical Representative
National Toxicology Program, NIEHS

[redacted]

3/20/12
Date

Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

[redacted]

4-2-12
Date

Study Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

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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 the potential of each test substance 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 Hershberger Bioassay (OPPTS 890.1400) is used as an *in vivo* screening assay for androgen agonists, androgen antagonists, and 5α-reductase inhibitors 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 two test substances selected by the National Toxicology Program for androgen agonist/antagonist activity and 5α-reductase inhibition properties using a castrated rat model (OPPTS 890.1400).

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 890.1400: Hershberger Bioassay (U.S. EPA), OECD Guideline 441 Hershberger Bioassay in Rats: A Short-term Screening Assay for (Anti) Androgenic Properties (adopted 7 September 2009) and ILS SOP's. 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.

Flutamide and testosterone propionate 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 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

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

[redacted]
Contract Office Technical Representative
NTP, NIEHS

National Toxicology Program Investigator

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

1.5 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.:
Facsimile No.:
E-mail:

1.6 Study Dates

Animal Arrival Dates:	29 March 2012
Experimental Start Date:	06 April 2012
Experimental Termination Date:	17 April 2012

TEST SUBSTANCES, REFERENCE SUBSTANCES, VEHICLE

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

CAS No.	27503-81-7
Source:	Sigma-Aldrich Company
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, 2012).

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 Reference Substance: Testosterone Propionate (Androgen agonist)

CAS No.	57-85-2
Source:	Sigma-Aldrich Company
Lot/Batch No.:	051M1803V
ILS Repository No.:	12-29
Formula:	$C_{22}H_{32}O_3$
Description:	White to off-white powder
Purity:	100%
Dose Formulation:	Testosterone propionate will be prepared at ILS in corn oil once at a dose level of 0.08 mg/mL and dispensed into vials to be used daily during the study.

Storage:

Reference Substance:	Room temperature, protected from light
Dose Formulation:	Between 1-10°C (Smith, 2011)

2.4 Reference Substance: Flutamide (Androgen antagonist)

CAS No.	13311-84-7
Source:	Sigma-Aldrich Company
Lot/Batch No.:	107K1293
ILS Repository No.:	11-77
Formula:	$C_{11}H_{11}F_3N_2O_3$
Description:	Yellow powder

Purity:	>99%
Dose Formulation:	Flutamide will be prepared at ILS in corn oil once at a dose level of 0.6 mg/mL and dispensed into vials to be used daily during the study.
Storage:	
Reference Substance:	Room temperature, protected from light
Dose Formulation:	Between 1-10°C
Stability:	
Dose Formulation:	Flutamide in corn oil stored between 1-10°C was demonstrated to be stable for 42 days (Graves, 2001).

2.5 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

2.6 Archive Samples

A ~1 g sample of the neat test substances, a ~1 mg sample reference substances (Flutamide Lot 107K1293 and testosterone propionate Lot 051M1803V), and 1 mL of the vehicle and dose formulations will be stored at room temperature until acceptance of the final report; after acceptance of the report by the sponsor dose formulation samples will be discarded.

2.7 Dose Formulation Analysis

Dose formulations will be prepared at ILS and analyzed at Research Triangle Institute (RTI) International and Midwest Research Institute (MRI) in accordance with GLP

regulations as promulgated by the U.S. EPA GLP Regulations (40 CFR Part 160). Three samples (top, middle, and bottom) of the test substance formulations 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 overnight to the following addresses for analysis prior to administration:

Ensulizole:
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

One hundred four castrated male Sprague-Dawley rats will be allocated to one of thirteen designated dose groups. To evaluate the test substances for agonist properties, animals will be administered one of two dose levels, or the vehicle control. To evaluate for antagonist properties animals will be administered one of three dose levels of the test substance and co-administered testosterone propionate (0.4 mg/kg, agonist). A vehicle control group will be administered corn oil and testosterone propionate (0.4 mg/kg) and serve as the positive control for the agonist group and the negative control for the antagonist group. Flutamide will be administered orally to animals that are co-administered with 0.4 mg/kg testosterone propionate and serve as a positive antagonist control.

Animals will be dosed for 10 consecutive days via oral gavage (test substances and flutamide) and subcutaneous injection (testosterone propionate) based upon daily body weights. Approximately 24-hours following the final dose administration, the animals will be humanely euthanized; the glans penis, ventral prostate, levator ani plus bulbocavernous muscle, Cowper's glands, and seminal vesicles with coagulating gland and fluid will be excised and weights recorded. Changes in androgen dependent tissue weights will be evaluated to determine the ability of the test substances to act as an androgen agonist/antagonist or 5α-reductase inhibitor.

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:	104/Castrated males. Surgical manipulation performed by Charles River Laboratories International, Inc.
Acclimation:	Animals will be allowed to recover from the surgical manipulation for at least five days at Charles River Laboratories International, Inc. The animals will then be acclimated to ILS for at least seven days in the room where the study will occur.
Age at administration:	Postnatal Day (PND) 59/60 Note: PND 0 is the day of birth
Weight at administration:	250-350 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 890.1400: Hershberger Bioassay (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*
	Autoclaved Purina 5L79 Rat and Mouse diet *ad libitum* given at Charles River Laboratories International, Inc. prior to shipment. 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.
Water:	Reverse osmosis treated tap water (City of Durham, NC) *ad libitum*
Supplied:	Glass water bottles with stainless steel sipper tube
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

ILS Project No. – Study No.:N135-248; The Hershberger Bioassay for Ensulizole and Avobenzone

Animal Room Conditions:

Temperature:	19-25°C
Humidity	30-70%
Lighting:	12/12 hour light/dark cycle

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 Designation

Table 1. Androgen Agonist

Group Number	Animal Identification	Test Substance/Controls	Test Substance Dose Level (mg/kg/day)
1	001-008	Corn Oil Control	0
2	009-016	Ensulizole	320
3	017-024	Ensulizole	1000
4	025-032	Avobenzone	320
5	033-040	Avobenzone	1000

Table 2. Androgen Antagonist

Group Number	Animal Identification	Test Substance/Controls	Test Substance Dose Level (mg/kg/day)
6*	041-048	Corn Oil Control + Testosterone Propionate	0 + 0.4
7	049-056	Ensulizole + Testosterone Propionate	100 + 0.4
8	057-064	Ensulizole + Testosterone Propionate	320 + 0.4
9	065-072	Ensulizole + Testosterone Propionate	1000 + 0.4
10	073-080	Avobenzone + Testosterone Propionate	100 + 0.4
11	081-088	Avobenzone + Testosterone Propionate	320 + 0.4
12	089-096	Avobenzone + Testosterone Propionate	1000 + 0.4
13	097-104	Flutamide + Testosterone Propionate	3.0 + 0.4

*Group will serve as the positive control for the androgen agonist assay

3.5 Dose Administration

The test substances, flutamide dose formulations, and the vehicle control dose formulations will be administered by oral gavage at a dosing volume of 5 mL/kg body weight. Testosterone propionate dose formulations will be administered by subcutaneous injection into the dorsoscapular region at a dosing volume of 0.5 mL/kg body weight. In co-administered animals, oral gavage will precede subcutaneous injections.

The dose formulations will be administered on a staggered start for 10 consecutive days (PND 59/60 through PND 68/69). The first four animals from each group will be dosed beginning on PND 59, and the second four animals from each group will begin on PND 60. Dosing will occur 24 hours (± 2 hours) from the previous dose. Dose volume will be determined on individual animal daily body weight. The dosing sequence will be

stratified across dosing 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 890.1400: Hershberger Bioassay (U.S. EPA, 2009).

3.5.2 Justification of Dose Levels

Selection of the highest dose level for each test substance was based upon the available LD50 and/or acute toxicity information in order to avoid death, severe suffering, or distress in the animals and second, takes into consideration available information on the doses used in other studies. In general, the highest dose should not cause a reduction in the final body weight of the animals greater than 10% of control body weight. The highest dose should ensure animal survival and that is without significant toxicity or distress to the animals after 10 consecutive days of administration up to a maximal dose of 1000 mg/kg/day.

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. If adverse clinical signs are seen additional observations may be recorded.
Preputial Separation (PPS):	Animals will be evaluated for preputial separation upon arrival. Separation not initiated, partial separation, a persistent thread of tissue between the glans penis and prepuce, or complete PPS will 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 by CO_2 asphyxiation with death confirmed by cervical dislocation; euthanasia will occur in the same order as they were dosed. The first four animals from each group will be euthanized on PND 69, and the second four animals from each group will be euthanized on PND 70.
Tissue Collection:	Gross observations of the tissues that are being excised for tissue weights will be recorded.
Tissue Weights:	The following tissues will be excised, trimmed of excess adhering tissue and fat, weighed, and weights recorded to the nearest 0.0001 g. 1. Ventral Prostate 2. Seminal vesicles with coagulating gland with fluid 3. Levator ani plus bulbocavernous muscle complex 4. Cowper's glands (weighed as a pair) 5. Glans penis.

3.8 Statistical Analysis

Descriptive statistics (mean, standard deviation, coefficient of variance) for initial and final body weight, body weight gain, and tissue weights will be analyzed using SAS (Cary, NC). Studentized residual plots will be used to detect possible 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 tissue weights will be analyzed by an ANOVA followed by pair wise comparisons using a Dunnett's t test (one tailed- tissues weights, two tailed- final body weight and body weight gain). Statistically significant effects will be reported when $p<0.05$. Positive controls will be analyzed by appropriate t-tests.

If preputial separation has not occurred in any of the groups, the incidence will be compared to the control group using a Fisher Exact test.

3.9 Performance Criteria

The study should be evaluated if 1) three or more of the ten possible individual CV's in the negative control and high dose group exceed the maximum allowable CV's designated for androgenic and anti-androgenic effects listed in Table 5, or 2) if at least two of the target tissues' p values fall between 0.05 and 0.10 when compared to the negative control.

Table 3. Maximum Coefficients of Variation

Tissue	Androgen Agonist	Androgen Antagonist
Glans Penis	22%	17%
Cowper's Glands	55%	35%
LABC	30%	20%
Ventral Prostate	45%	40%
Seminal Vesicles	40%	40%

Source: U.S. EPA (2009)

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.

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. (2012). 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.

Graves, S. (2001). Dose Formulation Development Study Report Flutamide. Study Project Number Project Number: G004110-AXG. Unpublished study report prepared by Battelle.

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

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

Smith, R. (2011). Storage Stability of Testosterone Propionate in Corn Oil. Unpublished study report prepared by Smithers Viscient, LLC. Study No. 13974.6106.

U.S. EPA (Environmental Protection Agency). (2009). Endocrine Disruptor Screening Program Test Guidelines. OPPTS 890.1400: Hershberger Bioassay. EPA 740-C-09-008. 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:

Appendix VIII:

Amendments, Deviations, and Notes to File

Integrated Laboratory Systems, Inc.

Protocol Amendment

ILS Project No.-Study No.:	N135-248
Protocol Amendment No.:	1
Section Amended:	2.7
Amendment Made:	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.
Sections Amended:	2.1, 2.2, 2.3, 2.4
Amendment Made:	All dose formulations will be stirred for at least 30 minutes prior to the start of dose administration and continuously stirred during dose administration.
Reason for Amendment:	Inclusion of instructions for handling of dose formulations.
Sections Amended:	2.4, 2.6
Amendment Made:	The lot number for Flutamide is 021M1406V.
Reason for Amendment:	Typographical error.

[redacted]
Chief, Toxicology Branch
National Toxicology Program, NIEHS

4/10/12
Date

[redacted]
Contract Office Technical Representative
National Toxicology Program, NIEHS

4/12/12
Date

[redacted]

4/13/12
Date

Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

[redacted]

4-13-12
Date

Study Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

ILS-A-066
Last Revised: 08/07/12

Integrated Laboratory Systems, Inc.
Protocol Deviation

ILS Project No.-Study No.:	N135-248
Protocol Deviation No.:	1
Protocol Section Deviated from:	3.1
Nature of Deviation:	Weight at dose administration was for animal 002 was slightly below (249.7g) the stated weight at dose administration (250-350g).
Reason for Deviation:	Animal was included to have enough animals on study.
Corrective Action:	None.
Impact on Study:	There is no significant impact on the study because all group means and standard deviations were not significantly different on the first day of dose administration and was the animals weight was below by <1 gram.
Protocol Section Deviated from:	3.2
Nature of Deviation:	Relative humidity was out of on the following date: 13 April 2012
Reason for Deviation:	Slight fluctuations in the HVAC system.
Corrective Action:	None, the HVAC system corrected the slightly lower humidity.
Impact on Study:	There is no significant impact on the study because the slightly lower humidity did not cause any abnormal clinical observations in the animals.
Protocol Section Deviated from:	3.5
Nature of Deviation:	The times recorded of dose administration for animals 080 and 088 were out of dose order.
Reason for Deviation:	It is likely that the incorrect hour was recorded, and not that the animals were dosed out of order.

Corrective Action:	Research assistants were reminded to appropriately record time of dose administration.
Impact on Study:	There is no significant impact on the study because the animals were administered dose formulations on these days, likely at the correct time, because the animals dosed before and after were recorded correctly.

[redacted]

8-7-12
Date

Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

Integrated Laboratory Systems, Inc.

SOP Deviation 1

ILS Project No.-Study No.: N135-248

SOP No.-Mod. No. Deviated: 718-10

SOP Section Deviated: II-A

Nature of Deviation: Morning room check was not performed on 06 April 2012.

Reason for Deviation: Technician oversight.

Corrective Action: Technicians were reminded to confirm all room checks are performed.

Impact on Study: None since all animals were observed as normal prior to dose administration on 06 April 2012.

[redacted]

Study Director, ILS, Inc.

5-3-12

Date

Integrated Laboratory Systems, Inc.

SOP Deviation 2

ILS Project No.-Study No.: N135-248

SOP No.-Mod. No. Deviated: 1119-11

SOP Section Deviated:	II-D-2
Nature of Deviation:	Cleaning of the gavage needles was not initialed and dated on the following dates: 09, 15 April 2012.
Reason for Deviation:	Technician oversight.
Corrective Action:	Technicians were reminded to initial and date after each task is performed.
Impact on Study:	None because it is likely that all gavage needles were cleaned, it was just not recorded.

[redacted]

5-3-12
Date

Study Director, ILS, Inc.