FINAL REPORT

Study Title

The Uterotrophic Assay (OPPTS 890.1600) with Oxybenzone, Octylmethoxycinnamate, Octylsalate and Octocrylene

ILS Project-Study Numbers

N135-231

Guideline Reference Number

OPPTS 890.1600

Author

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Performing Laboratory

Integrated Laboratory Systems, Inc.
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Sponsor

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

Date of Completion

01 September 2011

Revised: 20 April 2012 See Appendix X.

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Sponsor: National Institutes of Environmental Health

Sponsor Representative: [redacted]

Title: Contract Officer Technical Representative

Signature: [redacted]

Date: 9/1/11

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COMPLIANCE STATEMENT

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

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

Dose formulation analyses were performed at the following laboratories at the request of the sponsor: analysis for Octylmethoxycinnamate with [redacted] as the Study Director at Midwest Research Institute (Kansas City, MO), analysis for Oxybenzone with [redacted] as the Study Director, analysis for Octylsalate with [redacted] as the Study Director, and analysis for Octylcrylene with [redacted] as the Study Director, all at Battelle Memorial Institute (Columbus, OH).

[redacted]

9/1/11
Date

Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

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9/1/11
Date

Contract Officer Technical Representative
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This final report has been reviewed by:

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9/1/11
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Principal Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc

QUALITY ASSURANCE INSPECTION STATEMENT

Laboratory Project ID - Study No.: N135-231
Study Title: The Uterotrophic Assay (OPPTS 890.1600) with Oxybenzone, Octylmethoxycinnamate, Octylsalate and Octocrylene

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	02 May 2011	05 May 2011/05 May 2011
Necropsy	19 May 2011	19 May 2011/19 May 2011
Data Audit	15-17 June 2011	17 June 2011/20 June 2011
Draft Report	05, 08-09 August 2011	09 August 2011/ 11 August 2011
Final Report	30 August 2011	30 August 2011/ 30 August 2011

[redacted] _____ 09/01/2011
Quality Assurance Officer Date

TABLE OF CONTENTS

Tables:

Appendices:

Study Summary

Ovariectomized adult female rats were orally administered corn oil (vehicle control), 320 or 1000 mg/kg oxybenzone, octylmethoxycinnamate, octylsalate, octocrylene or 0.1 mg/kg 17α-Ethinyl estradiol (positive control) for three consecutive days and then humanely euthanized. Body weights and clinical observations were performed daily. At termination, uteri were excised and wet and blotted weights recorded.

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

Administration of oxybenzone significantly decreased body weight gain at 1000 mg/kg, but not at 320 mg/kg compared to vehicle control animals. Octylsalate (1000 mg/kg) significantly decreased final body weight and body weight gain (320 and 1000 mg/kg) compared to the control group. Administration of either octylmethoxycinnamate or octocrylene did not affect body weights or body weight gain compared to vehicle controls. Uterine weights (wet and blotted) were not significantly different at either dose level of oxybenzone, octylsalate, octylmethoxycinnamate or octocrylene compared to the vehicle control.

Oral administration of oxybenzone, octylsalate, octylmethoxycinnamate or octocrylene, up to the limit dose level of 1000 mg/kg, are not estrogenic in the ovariectomized rat model Uterotrophic Assay (OPPTS 1600).

INTRODUCTION

1.1 Study Title

The Uterotrophic Assay (OPPTS 890.1600) with Oxybenzone, Octylmethoxycinnamate, Octylsalate and Octocrylene

1.2 Laboratory Project Identification

ILS Project No.-Study No.: N135-231

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

1.4 Purpose of the Study

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

1.5 Sponsor

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

NIEHS Investigator

[redacted]
Telephone No.:
E-mail:

Study Monitor

[redacted]
Contract Officer Technical Representative

Telephone No.:
E-mail:

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

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

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

Study Director

Telephone No.:
Facsimile No.:
E-mail:

1.7 Study Dates

Study Initiation Date: 06 May 2011
Animal Arrival Date: 09 May 2011
Experimental Start Date: 16 May 2011
Experimental End Date: 20 May 2011

TEST SUBSTANCE

2.1 Test Substance **2-Hydroxy-4-methoxybenzone (Oxybenzone)**

Product Name: 2-Hydroxy-4-methoxybenzophenone

CAS No. 131-57-7

Source: Ivy Fine Chemical Corporation

Lot/Batch No.: 20080801

ILS Repository No.: 11-29

Formula: $C_{14}H_{12}O_3$

Description: Light yellow powder

Purity: 99.9%

Expiration Date: 01 August 2012

Dose Formulation: Test substance was prepared one time during the study at ILS. Oxybenzone formulation, in corn oil,

were prepared in glass bottles at concentrations of 64 and 200 mg/ml and dispensed into 15 mL amber vials that were used for daily dosing throughout the study.

Storage:

Test Substance: Room Temperature

Dose Formulation: Stored between 1 and 10°C and protected from light

Stability:

Dose Formulation: Dose formulations prepared in corn oil and held at approximately 5 and 25°C for 42 days were considered stable (Richey, 2011c).

2.2 Test Substance **2-Ethylhexyl p-methoxycinnamate (Octylmethoxycinnamate)**

Product Name: Octyl 4-methoxycinnamate

CAS No. 5466-77-3

Source: Acros Organics

Lot/Batch No.: A0293319

ILS Repository No.: 11-32

Formula: $C_{18}H_{26}O_3$

Description: Clear colorless liquid

Purity: 99.8%

Expiration Date: 04 July 2011

Dose Formulation: Test substance was prepared one time during the study at ILS. Octylmethoxycinnamate formulations, in corn oil, were prepared in glass bottles at concentrations of 64 and 200 mg/ml and dispensed into 15 mL amber vials that were for daily dosing throughout the study.

Storage:

Test Substance: Room Temperature

Dose Formulation: Stored between 1 and 10°C and protected from light

Stability:

Dose Formulation: Dose formulation prepared incorn oil and held up to 60°C for 14 days were considered stable (Kroenke, 2011).

2.3 Test Substance **Octyl Salicylate (Octylsalate)**

Product Name: 2-Ethylhexyl salicylate

CAS No. 118-60-5

Source: Sigma Aldrich

Lot/Batch No.: 44698PJ

ILS Repository No.: 11-30

Formula: $C_{15}H_{22}O_3$

Description: Colorless liquid

Purity: 99.6%

Expiration Date: 12 August 2011

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

Storage:

Test Substance: Room Temperature

Dose Formulation: Stored between 1 and 10°C and protected from light

Stability:

Dose Formulation:	Dose formulations prepared in corn oil held at approximately 5 and 25°C for 42 days were considered stable (Richey, 2011b).

2.4 Test Substance **2-Ethylhexyl 2-Cyano-3,3-diphenylacrylate (Octocrylene)**

Product Name:	2-Ethylhexyl 2-Cyano-3,3-diphenylacrylate
CAS No.	6197-30-4
Source:	Sigma Aldrich
Lot/Batch No.:	01697MJ
ILS Repository No.:	11-31
Formula:	$C_{24}H_{27}NO_2$
Description:	Yellow viscous liquid
Purity:	99.2%
Expiration Date:	11 August 2012
Dose Formulation:	Test substance was prepared one time at ILS during the study. Octocrylene formulations, in corn oil, were prepared in glass bottles at concentrations of 64 and 200 mg/ml and dispensed into 15 mL amber vials that were used for daily dosing throughout the study. Dose concentrations were adjusted to correct for purity of octocrylene.
Storage:	
Test Substance:	Room Temperature
Dose Formulation:	Stored between 1 and 10°C and protected from light
Stability:	
Dose Formulation:	Dose formulations prepared in corn oil held and at approximately 5 and 25°C for 42 days were considered stable (Richey, 2011a).

2.5	**Reference Substance:**	**17α-Ethinyl Estradiol**
	CAS No.	57-63-6
	Source:	Sigma Aldrich
	Lot/Batch No.:	090M1241V
	ILS Repository No.:	11-40
	Formula:	$C_{20}H_{24}O_2$
	Description:	White powder
	Purity:	≥98%
	Expiration Date:	February 2012
	Dose Formulation:	ILS prepared 17α-Ethinyl estradiol in corn oil once at a dose level of 0.02 mg/mL and aliquoted into amber vials to be used daily during the study.
	Storage:	
	Reference Substance:	Room temperature and protected from light
	Dose Formulation:	Stored between 1-10°C
	Stability:	
	Dose Formulation:	17α-Ethinyl estradiol in corn oil, stored between 1-10°C, is stable for 42 days (Messer, 2002).
2.6	**Vehicle**	**Corn Oil**
	CAS No.:	8001-30-7
	Source:	MP Biomedicals, LLC (Solon, OH)
	Lot/Batch No.:	7862K
	ILS Repository No.:	11-94
	Formula:	$C_{27}H_{50}O_6$
	Description:	Yellow oil

Storage:

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

2.7 Archival Samples

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

2.8 Dose Formulation Analysis

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

Octylmethoxycinnamate:
Midwest Research Institute
Program: NTP Chemistry Support
425 Volker Boulevard
Kansas City, MO 64110-2299

Oxybenzone, Octylsalate and Octocrylene:
Battelle Memorial Institute
TOXBC Test Article Custodian
651 W. Fifth Avenue
Columbus, OH 43201-2693

Samples of dose formulations prepared on 04 and 05 May 2011 were collected from the top, middle, and bottom of the formulation and sent to Midwest Research Institute or Battelle Memorial Institute for analysis. Midwest Research Institute or Battelle Memorial Institute analyzed samples in duplicate for concentration and homogeneity (Appendix II).

Concentration results were acceptable if the mean concentration was within 10% of the target concentration. Homogeneity results were acceptable if the coefficient of variation was less than ≤5% of the target concentration.

EXPERIMENTAL DESIGN

3.1 Test System

Species:	Rat, *Rattus norvegicus*
Strain:	Sprague-Dawley Crl:CD®(SD) IGS
Source:	Charles River Laboratories International, Inc. (Raleigh, NC)
Number/Sex:	80/Ovariectomized females. Surgical manipulation performed by Charles River Laboratories International, Inc.
Date of birth:	21 March 2011
Age at arrival:	Postnatal Day (PND) 49 Note: PND is the date of birth
Acclimation:	Animals were acclimated in the study room for 7 days
Age at dose administration:	PND 56
Weight at dose administration:	232.3 – 282.8 grams
Identification:	Each animal was uniquely identified by ear punch prior to dose administration. Until the animals were ear punched, they were identified by the temporary numbers located on the animal's cage.
Justification:	Animal model used is in accordance with OPPTS 890.1600: Uterotrophic Assay (U.S. EPA, 2009) and OECD Guideline 440 (OECD, 2007). The EPA test guideline prefers the use of the ovariectomized rat model compared to the immature rat model, while the OECD guideline does not state a preference.

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, 1996).

Housing (pre-allocation):	1 per cage
Housing (post allocation):	2 per cage
Cage Changes:	Twice per week
Cage Type:	Polycarbonate with micro-isolator top
Cage Size:	23 cm wide by 44 cm long (1012 cm^2 area) and 21 cm high
Bedding:	Absorbent heat-treated hardwood bedding (Northeastern Products Corp., Warrensburg, NY)
Diet:	Teklad Global 16% Protein Rodent Diet (Teklad Diets, Madison WI) *ad libitum* Prior to shipment rats were given Autoclaved Purina5L79 Rat and Mouse diet *ad libitum* at Charles River Laboratories International, Inc. A copy of the diet composition is included in the raw data.
Analysis:	The manufacturer's analytical results are included in the raw data and reviewed prior to animal arrival. The total genistein equivalent of genistein plus daidzein (as described by Owens et al., 2003) was determined to be 8.0 µg/g.
Water:	Reverse osmosis treated tap water (City of Durham, NC) *ad libitum*
Supplied:	Glass water bottles with stainless steel sipper tubes
Analysis:	The results of the current annual comprehensive chemical analyses of water from National Testing Laboratories, Inc. (Cleveland, OH) were reviewed prior to initiation of the study and are included in the raw data.
Water Bottle Changes:	Once per week

Animal Room Conditions:

Temperature:	21-24°C
Humidity	34-46%
Lighting:	12/12 hour light/dark cycle
Cleaning:	Sanitized within 4 days of receipt

Enrichment: None

STUDY DESIGN

4.1 Allocation

The animals were assigned to a dose group using a procedure that stratifies animals across groups by body weight such that mean body weight of each group was not statistically different from any other group using analysis of variance [ANOVA, Statistical Analysis System (SAS) version 9.1, SAS Institute, Cary, NC]. Only clinically healthy animals were used in the study.

4.2 Group Designation

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

Group Number	Animal Identification	Dose Group	Nominal Dose Level (mg/kg/day)
1	01-08	Vehicle Control (corn oil)	0
2	09-16	Oxybenzone	320
3	17-24	Oxybenzone	1000
4	25-32	Octylmethoxycinnamate	320
5	33-40	Octylmethoxycinnamate	1000
6	41-48	Octylsalate	320
7	49-56	Octylsalate	1000
8	57-64	Octocrylene	320
9	65-72	Octocrylene	1000
10	73-80	17α-Ethinyl estradiol	0.1

4.3 Dose Administration

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

4.3.1. Justification of Route of Administration

Selection of the route of administration is in accordance with OPPTS 890.1600: Uterotrophic Assay (U.S. EPA, 2009) and OECD Guideline 440 (OECD, 2007).

4.3.2. Justification of Dose Levels

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

A dose level of 0.1 mg/kg 17α-Ethinyl estradiol was determined based on results presented by Laws et al. (2000).

4.3.3. Disposal of Dose Formulations

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

4.4 In-Life Animal Observations

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

4.5 Termination

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

The ends of the uterine horns were examined for the presence of any ovarian tissue. If ovarian tissue was observed it was noted in the study records.

Gross observations of the uterus were recorded.

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

1. Wet uterus
2. Blotted uterus

4.6 Statistical Analysis

Descriptive statistics (mean and standard deviation) were calculated using MS Excel. Final body weight, body weight gain, and tissue weights were analyzed using SAS version 9.1 (SAS Institute, Cary, NC). Studentized residual plots were used to detect possible outliers and Levene's test was used to assess homogeneity of variance.

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

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

RESULTS

5.1 Dose Formulation Analysis

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

Table 2. Dose Formulation Results

Dose Group	Nominal Dose Concentration (mg/mL)	Actual Dose Concentration* (mg/mL) [Percent from Nominal]	Percent CV* (Homogeneity)	Nominal Dose Level (mg/kg/day)	Actual Dose Level (mg/kg/day)
Oxybenzone†	64	63.9 [0.2]	0.3	320	319.5
Oxybenzone†	200	211 [5.5]	1.2	1000	1055.0
Octylmethoxycinnamate¥	64	65.2 [2.0]	3.2	320	326.0
Octylmethoxycinnamate¥	200	202.9 [1.4]	2.4	1000	1014.5
Octylsalate†	64	63.1 [1.4]	0.7	320	315.5
Octylsalate†	200	192 [4.0]	0	1000	960.0
Octocrylene†	64	60.7 [5.2]	1.2	320	303.5
Octocrylene†	200	185 [7.5]	3.0	1000	925.0

Preparation Dates: 04 May¥ and 05 May† 2011
*Sources: Hainey, 2011; Kerns, 2011; Richey, 2011d,e
Abbreviations: CV – coefficient of variation

5.2 In-Life Animal Observations

Mortality/Moribundity

Animals administered corn oil (vehicle control), oxybenzone, octylmethoxycinnamate, octylsalate (320 mg/kg) and octocrylene survived to the scheduled study termination with no animals showing signs of moribundity.

After the third day of administration of 1000 mg/kg octylsalate, two of eight animals were found dead. A dosing error was ruled out as the cause of death.

Clinical Observations

Animals administered corn oil (vehicle control), oxybenzone, octylmethoxycinnamate, octylsalate, or octocrylene exhibited no abnormal clinical signs. (Appendix IV).

Cage-side Observations

Animals administered corn oil, oxybenzone, octylmethoxycinnamate, or octocrylene exhibited no abnormal post-dose clinical signs. Animals administered 320 mg/kg octylsalate did not exhibit any abnormal post-dose clinical signs however, one of six surviving rats administered 1000 mg/kg octylsalate exhibited uncoordinated movement and hunched posture prior to euthanasia (study day 4) (Appendix IV).

Body Weights

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

The body weight gain of female rats administered 1000 mg/kg oxybenzone was significantly decreased compared to the vehicle control group.

Final body weight (88.9% of control weight) of rats administered 1000 mg/kg octylsalate and body weight gain of rats administered 320 mg/kg and 1000 mg/kg were significantly decreased compared to the vehicle control group.

Final body weights (and body weight gain) of rats administered 320 mg/kg oxybenzone, octylmethoxycinnamate and octocrylene (320 and 1000 mg/kg) were not statistically different compared to the vehicle control group.

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

Dose Group	Dose Level (mg/kg/day)	n	Initial Mean Body Weight (g) ± SD	Final Mean Body Weight (g) ± SD	Mean Body Weight Change (g) ± SD
Vehicle Control	0	8	257.7 ± 12.9	268.1 ± 10.2	10.4 ± 6.6
Oxybenzone	320	8	257.8 ± 13.4	268.8 ± 13.1	11.1 ± 2.9
Oxybenzone	1000	8	256.2 ± 11.6	254.6 ± 9.4	**-1.6 ± 5.8***
Octylmethoxycinnamate	320	8	260.7 ± 10.6	271.5 ± 9.0	10.8 ± 6.3
Octylmethoxycinnamate	1000	8	259.5 ± 14.5	264.8 ± 12.0	5.3 ± 6.3
Octylsalate	320	8	260.3 ± 11.4	261.1 ± 13.1	**0.8 ± 5.7***
Octylsalate	1000	8	257.6 ± 9.6	**238.5 ± 16.6[1]***	**-16.1 ± 8.6[1]***
Octocrylene	320	8	255.5 ± 11.3	267.6 ± 11.1	12.0 ± 2.8
Octocrylene	1000	8	259.2 ± 10.6	267.3 ± 9.9	8.1 ± 6.1

Abbreviation: SD- standard deviation

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

[1]Mean calculated from 6 animals, two animals died on study day 3

5.3 Necropsy Procedures

Uterine Weights

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

Administration of oxybenzone, octylmethoxycinnamate, octylsalate or octocrylene did not affect either wet or blotted uterine weights compared to the vehicle control group. The positive control, 17α-Ethinyl estradiol, resulted in significantly increased wet and blotted uterine weights compared to the vehicle control group.

Table 4. Uterine Weights

Dose Group	Dose Level (mg/kg/day)	n	Uterine Weight-Wet (g) Mean ± SD	Uterine Weight-Blotted (g) Mean ± SD
Vehicle Control	0	8	94.3. ± 14.5	86.8 ± 13.7
Oxybenzone	320	8	94.3 ± 11.2	87.5 ± 10.5
Oxybenzone	1000	8	103.8 ± 19.0	96.3 ± 17.9
Octylmethoxycinnamate	320	8	89.1 ± 11.2	82.2 ± 11.5
Octylmethoxycinnamate	1000	8	94.4 ± 9.5	87.5 ± 9.7
Octylsalate	320	8	92.9 ± 13.1	86.5 ± 12.9
Octylsalate[1]	1000	6	94.5 ± 8.6	87.0 ± 8.2
Octocrylene	320	8	86.5 ± 9.7	80.0 ± 10.0
Octocrylene	1000	8	91.4 ± 6.4	85.3 ± 6.0
17α-Ethinyl estradiol†	0.1	8	**277.1 ± 76.3**†	**210.1 ± 27.5**†

Abbreviations: SD- standard deviation

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

[1]Mean calculated from 6 animals, two animals died on study day 3

5.4 Performance Criteria

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

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

SUMMARY

Ovariectomized adult female rats were orally administered corn oil, oxybenzone, octylmethoxycinnamate, octylsalate, octocrylene or EE (positive control) for three consecutive days and then euthanized. Body weights and clinical observations were performed daily. At termination, uteri were excised and wet and blotted weights recorded.

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

Administration of oxybenzone significantly decreased body weight gain at a dose level of 1000 mg/kg, but not at 320 mg/kg. Administration of octylsalate significantly decreased final body weight and body weight gain at a dose level of 1000 mg/kg, and body weight gain at 320 mg/kg.

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

Oral administration of oxybenzone, octyl salate, octylmethoxycinnamate or octocrylene, up to the limit dose level of 1000 mg/kg, were not estrogenic in the ovariectomized rat model Uterotrophic Assay (OPPTS 890.1600).

REFERENCES

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Haney, R. (2011) Formulation analysis of 2-Hydroxy-4-methoxyoxybenzophenone (HMB) in corn oil. Battelle Project No: G006623-EEB. NTP ChemTask No: CHEM11267. Unpublished study report prepared by Battelle.

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Richey, J. (2011b) Dose formulation developmental study report Octyl Salicylate. Battelle Project No: G005430-DZZ. NTP ChemTask No: CHEM10925. Unpublished study report prepared by Battelle.

Richey, J. (2011c) Dose formulation developmental study report 2-Hydroxy-4-methoxybenzophenone. Battelle Project No: G005430-EAB. NTP ChemTask No: CHEM10928. Unpublished study report prepared by Battelle.

Richey, J. (2011d) Formulation analysis of 2-Ethylhexyl 2-Cyano-3,3-diphenylacrylate (Octocrylene) in corn oil. Battelle Project No: G006623-EEB. NTP ChemTask No: CHEM11269. Unpublished study report prepared by Battelle.

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KEY PERSONNEL

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

Appendix I:

Certificate of Analysis

N135-231
[redacted] 5-5-11

IVYCHEM

IVY FINE CHEMICALS CORPORATION
http://www.ivychem.com

CERTICATE OF ANALYSIS

Product Name:	**2-HYDROXY-4-METHOXYBENZOPHENONE**		
Catalog Number	HH13-026		
CAS Number	131-57-7		
Batch No.:	20080801	**Quantity:**	1000 KG
Manu. Date:	August 5, 2008	**Expiry Date:**	August 4, 2010
Date of Report:	August 5, 2008	**Package:**	
Quality Specifications:	Specifications (In house)		

Test	Standard	Results
Appearance	Light yellow powder	Light yellow powder
Assay (GC)	98.0% min	99.9%
Melting Point	62 °C to 65 °C	63.5 °C to 64.8 °C
Conclusion: **Conform**		

Note: Quantity specified in Certificate of Analysis refers to the batch size of the lot produced by the supplier, not the quantity procured.

Battelle Study No. G005430-DID 4
Revised

N135-23[redacted] 5-23-11

Certificate of Analysis

SIGMA-ALDRICH

Product Name	2-Ethylhexyl 2-cyano-3,3-diphenylacrylate, 97%
Product Number	415820
Product Brand	ALDRICH
CAS Number	6197-30-4
Molecular Formula	$(C_6H_5)_2C=C(CN)CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3$
Molecular Weight	361.48

TEST	SPECIFICATION	LOT 01697MJ RESULTS
Appearance (Color)	Yellow	Yellow
Appearance (Form)	Viscous Liquid	Viscous Liquid
Infrared spectrum	Conforms to Structure	Conforms
Purity (GC)	≥96.5 %	99.2 %
Specification Date:		OCT 2008
Date of QC Release:		OCT 2008
Print Date:		OCT 22 2008

[redacted], Supervisor
Quality Control
Milwaukee, Wisconsin USA

~~A135-21~~ (A)

N135-231 [redacted] 5-23-11

Certificate of Analysis

SIGMA-ALDRICH

Product Name	2-Ethylhexyl salicylate, ≥98%
Product Number	W514500
Product Brand	ALDRICH
CAS Number	118-60-5
Molecular Formula	$(HO)C_6H_4CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3$
Molecular Weight	250.33

TEST	SPECIFICATION	LOT 44698PJ RESULTS
Appearance (Color)	Colorless	Colorless
Appearance (Form)	Liquid	Liquid
Refractive Index at 20 ° C	1.500 - 1.504	1.502
Infrared spectrum	Conforms to Structure	Conforms
Purity (GC)	≥99.0 %	99.6 %
Color Test	≤100 APHA	10 APHA
Arsenic (As)	≤3.0 ppm	< 1.0 ppm
Cadmium (Cd)	≤1.0 ppm	< 1.0 ppm
Mercury (Hg)	≤1.0 ppm	< 1.0 ppm
Lead (Pb)	≤10.0 ppm	< 1.0 ppm
Specification Date:		DEC 2008
Date of QC Release:		DEC 2008
Print Date:		DEC 18 2008

[redacted]

[redacted]: Supervisor
Quality Control
Milwaukee, Wisconsin USA

(A)(1) [redacted] 5-23-11

CERTIFICATE OF ANALYSIS

Product 29116 **Octyl 4-methoxycinnamate,98%,stabilized**

Specifications

Appearance	CLEAR COLOURLESS TO YELLOW LIQUID
Infrared spectrometry	AUTHENTIC
Separat. techn. GC	>97.5 %
Acid value	<1 mg KOH/g
Specific abs. A (1%/1cm)	>830 (at 307 to 308 nm in methanol)
Specific gravity	(25/25°C) 1.007 to 1.012
Refractive Index	1.5430 to 1.5470 (20°C, 589 nm)
Stabilizer	0.05 to 0.1 % BHT

General Product Data

Version	00
CAS No.	5466-77-3
Molecular weight	290.39
Molecular formula	C18 H26 O3
Linear formula	
Flash point (°C)	193

Lot Specific Data for Lot No.: A0293319

Appearance	CLEAR COLOURLESS LIQUID
Infrared spectrometry	AUTHENTIC
Separat. techn. GC	99.8 %
Acid value	0.1 mg KOH/g
Specific abs. A (1%/1cm)	865 (at 307 to 308 nm in methanol)
Specific gravity	(25/25°C) 1.0096
Refractive Index	1.5453 (20°C, 589 nm)
Stabilizer	0.09 % BHT

NI35-231
5-5-11

ACROS ORGANICS

[redacted]

Issued: 10-08-10

[redacted] **Quality Assurance Manager**

Acros Organics Geel West Zone 2, Janssen Pharmaceuticalaan 3a, B-2440 Geel, Belgium Tel +32 14/57.52.11 - Fax +32 14/59.34.34 Internet: http://www.acros.com
1 Reagent Lane, Fair Lawn, NJ 07410,USA Fax 201-796-1329

N135-231 [redacted] 5-23-11

IVYCHEM

IVY FINE CHEMICALS

http://www.ivychem.com

CERTICATE OF ANALYSIS

Product Name	**2-HYDROXY-4-METHOXYBENZOPHENONE**		
Synonym	Oxybenzone		
Catalog Number	HH13-026		
CAS Number	131-57-7		
Batch Number	20100801	**Quantity**	200 KG
Manu. Date	August 2, 2010	**Expiry Date**	August 1, 2012
Date of Report	August 2, 2010	**Package**	
Quality Specifications	Specifications (In house)		

Test	**Standard**	**Results**
Appearance	Light yellow to green crystalline powder	Light yellow crystalline powder
Assay (HPLC)	98% min	99.92%
Melting Point	62 °C to 65 °C	63.8 °C to 64.8 °C
Loss on Drying	0.5% max	0.07%
Heavy Metals	<= 5 ppm	2.9 ppm
Conclusion: **Conform**		

N135-231
[redacted] 5-5-11

Certificate of Analysis

SIGMA-ALDRICH

Product Name	2-Ethylhexyl 2-cyano-3,3-diphenylacrylate, 97%
Product Number	415820
Product Brand	ALDRICH
CAS Number	6197-30-4
Molecular Formula	$(C_6H_5)_2C=C(CN)CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3$
Molecular Weight	361.48

TEST	SPECIFICATION	LOT 01697MJ RESULTS
Appearance (Color)	Yellow	Yellow
Appearance (Form)	Viscous Liquid	Viscous Liquid
Infrared spectrum	Conforms to Structure	Conforms
Purity (GC)	≥96.5 %	99.2 %
Specification Date:		OCT 2008
Date of QC Release:		OCT 2008
Print Date:		OCT 22 2008

[redacted]
Quality Control
Milwaukee, Wisconsin USA

http://www.sigmaaldrich.com/catalog/CertOfAnalysisPage.do?symbol=415820&LotNo=01697MJ... 8/30/2010

n135-231
[redacted] 5-5-11

Certificate of Analysis

SIGMA-ALDRICH

Product Name	2-Ethylhexyl salicylate, ≥99%
Product Number	W514500
Product Brand	ALDRICH
CAS Number	118-60-5
Molecular Formula	$(HO)C_6H_4CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3$
Molecular Weight	250.33

TEST	SPECIFICATION	LOT 44698PJ RESULTS
Appearance (Color)	Colorless	Colorless
Appearance (Form)	Liquid	Liquid
Refractive index at 20 °C	1.500 - 1.504	1.502
Infrared spectrum	Conforms to Structure	Conforms
Purity (GC)	≥99.0 %	99.6 %
Color Test	≤100 APHA	10 APHA
Arsenic (As)	≤3.0 ppm	< 1.0 ppm
Cadmium (Cd)	≤1.0 ppm	< 1.0 ppm
Mercury (Hg)	≤1.0 ppm	< 1.0 ppm
Lead (Pb)	≤10.0 ppm	< 1.0 ppm
Specification Date:		DEC 2008
Date of QC Release:		DEC 2008
Print Date:		DEC 18 2008

[redacted]
[redacted] Supervisor
Quality Control
Milwaukee, Wisconsin USA

N135-231
[redacted] 5-5-11

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

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

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

Certificate of Analysis

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

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

Test	Specification	Result
Identity	Passes	Passes

Color (Lovibond): 1.6

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

This is an electronically generated document
mailto:biotech@mpbio.com
http://www.mpbio.com
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)

Appendix II:

Dose Formulation Analysis

Battelle
The Business of Innovation

BATTELLE-FA

Analytical Chemistry Services for the NTP
NIH Contract No.: HHSN273201000016C
Battelle Project No.: G006623-EEB
NTP ChemTask No.: CHEM11267
CAS No.: 131-57-7

FORMULATION ANALYSIS OF
2-HYDROXY-4-METHOXYBENZOPHENONE (HMB) IN CORN OIL

June 9, 2011

Prepared By:

[redacted]
Study Director

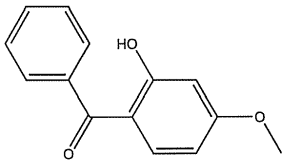
Approved By:

[redacted]
Steven W. Graves, B.S.
Principal Investigator

Submitted to:

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

FORMULATION ANALYSIS OF
2-HYDROXY-4-METHOXYBENZOPHENONE (HMB) IN CORN OIL

<table>
<tr><td colspan="2">CAS No.: 131-57-7</td><td>Lot No.: 20100801 [Ivy Fine Chemicals, 99.92% pure by high performance liquid chromatography (HPLC)]</td></tr>
<tr><td colspan="2">Battelle Chemical ID Code: 292</td><td>Samples Analyzed:
<u>Batch</u> — <u>Concentration</u>
N135-11-94-5511A — 0 mg/mL
11-29-1 T — 20 mg/mL
11-29-1 M — 20 mg/mL
11-29-1 B — 20 mg/mL
11-29-2 T — 64 mg/mL
11-29-2 M — 64 mg/mL
11-29-2 B — 64 mg/mL
11-29-3 T — 200 mg/mL
11-29-3 M — 200 mg/mL
11-29-3 B — 200 mg/mL</td></tr>
<tr><td colspan="2">Battelle Task No.: 16-292-FA-314</td><td>Sample Receipt Dates: 5/6 and 5/9/11</td></tr>
<tr><td colspan="2">NTP Task No.: CHEM11267</td><td>Submitter: Integrated Laboratory Systems, Inc. (ILS)</td></tr>
<tr><td colspan="2">Program Supported: TOX</td><td>Study Lab: ILS</td></tr>
<tr><td colspan="2">Analysis Dates: 5/9-5/10/11</td><td>Mix Date: 5/5/11</td></tr>
<tr><td colspan="2">Interim Results Date: 5/11/11</td><td>Receipt Condition: Good</td></tr>
<tr><td colspan="2">SOPs: CSCSPEC.II-051-00, Standard Operating Procedure for the Analysis of 2-Hydroxy-4-Methoxybenzophenone (HMB) Formulations in Corn Oil</td><td>Shipping Container: Total of ten amber glass vials
Storage Conditions (@ Battelle): Room Temperature (~25°C)</td></tr>
<tr><td>Structure
 
</td><td>Mol. Wt.
228.25 g/mol</td><td>Mol. Formula
$HOC_6H_3(OCH_3)COC_6H_5$</td></tr>
</table>

EXECUTIVE SUMMARY

Formulations of 2-hydroxy-4-methoxybenzophenone (HMB) in corn oil at target concentrations of 0, 20, 64, and 200 mg/mL were prepared by ILS and analyzed by Battelle to determine their concentration and homogeneity prior to administration in support of a TOX study.

The concentrations of all formulations containing HMB were within 10 percent of target, the National Toxicology Program (NTP) acceptance limit. The relative standard deviation (RSD) values were also within the specified acceptance limit. They also met the acceptance criteria for homogeneity. The 0 mg/mL formulation contained no detectable HMB. All other quality criteria stated in the SOP were within acceptance limits.

QUALITY ASSURANCE STATEMENT

FORMULATION ANALYSIS OF
2-HYDROXY-4-METHOXYBENZOPHENONE (HMB) IN CORN OIL

NTP ChemTask No.: CHEM11267
Battelle Project No.: G006623-EEB
Battelle Task No.: 16-292-FA-314

Listed below are the phases and/or procedures performed by Battelle that were reviewed by the Quality Assurance Unit (QAU) during performance of the task described in this report. Adverse findings, if any, were reported to the Study Director at the time of review.

Critical Phase Inspected	Date Inspected	Date Reported to Study Director and Management
Formulation analysis	5/9/11	5/10/11
Audit study file	5/24/11	5/24/11
Audit final analytical report	5/24/11	5/24/11

This report reflects the procedures and raw data generated in this study.

In addition to the study-specific audits/inspections cited above, routine inspections of the general facilities and equipment were performed by the QAU and reports were submitted to management as follows:

Facility/Equipment	Date Inspected	Date of Report to Management
Chemistry Technical Center Inspection	12/2, 12/15/08	12/2, 12/22/08
	12/16, 12/23/09	12/16, 12/31/09
	12/28, 12/30/10	12/30/10

[redacted] 6-1-11
Quality Assurance Unit Date

COMPLIANCE STATEMENT

This study was conducted in accordance with the Food and Drug Administration's (FDA's) Good Laboratory Practice (GLP) regulations (21 CFR, Part 58), with the exception of archival of study records at the close of the study. These records are gathered, microfiched, and archived periodically for finalized studies for this program.

[redacted] 6/9/11

Study Director Date

Date Study Initiated (Date Protocol Signed): May 4, 2011
Date Study Completed (Date Final Report Signed): June 9, 2011

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

This report contains:

- A description of the analyses of the formulations for concentration and homogeneity
- Results from the analysis
- Figures
- Conclusions.

This work was performed at Battelle, 505 King Avenue, Columbus, OH 43201, and supports a TOX study.

2.0 FORMULATION SAMPLES

Formulation samples prepared in corn oil (approximately 10 mL each) were received from ILS on May 6 and May 9, 2011. The samples were formulated on May 5, 2011 with an expiration date of June 17, 2011. They were identified as being from ILS Protocol No. N135-231/232 and had the following concentrations and log numbers.

Table 1. Formulation Samples

Concentration (mg/mL)	ILS Log No.
0	N135-11-94-5511A
20	11-29-1 T
20	11-29-1 M
20	11-29-1 B
64	11-29-2 T
64	11-29-2 M
64	11-29-2 B
200	11-29-3 T
200	11-29-3 M
200	11-29-3 B

All samples that were supplied by ILS in Table 1 were analyzed.

3.0 FORMULATION ANALYSIS FOR CONTENT AND HOMOGENEITY

The formulations were analyzed for HMB according to CSCSPEC.II-051-00, "Standard Operating Procedure for the Analysis of 2-Hydroxy-4-Methoxybenzophenone (HMB) Formulations in Corn Oil." This SOP was based on work originally conducted under the preliminary chemical studies (PCS) task for

HMB, Battelle Study No. G005430-DYS, NTP ChemTask No. CHEM10881 and the dose formulation development (DFD) task for HMB in corn oil, Battelle Study No. G005430-EAB, NTP ChemTask No. CHEM10928. The experimental limit of quantitation (ELOQ) is 0.01 mg/mL, which is the nominal concentration of the lowest standard for this task. This section describes the method, results, and conclusions.

3.1 Preparation of Diluted Vehicle Solution

The diluted vehicle solution was prepared by adding 1 mL of corn oil to a 100-mL volumetric flask and dissolving it in and diluting the flask to volume with acetone. The flask was sealed and the contents mixed well.

3.2 Preparation of Internal Standard (IS)

IS solution was prepared by weighing approximately 250 mg of benzophenone into a 50-mL volumetric flask and dissolving it in and diluting the flask to volume with acetone. The flask was sealed and the contents mixed well.

3.3 Preparation of Standards and Blanks

3.3.1 Stocks

The amounts of HMB shown in Table 2 were weighed into individual 50-mL volumetric flasks. The chemical was dissolved in and the flask diluted to volume with acetone. The flasks were sealed and the contents mixed well.

Table 2. Preparation of Stocks

ID	Target Concentration (mg/mL)	Target Weight (mg)
A	1.25	62.5 ± 2
B	1	50 ± 2

3.3.2 Spiking Solutions

The volumes of A and B indicated in Table 3 were pipetted into individual volumetric flasks. The flasks were diluted to volume with acetone, sealed, and the contents mixed well. A single solution was prepared at all concentrations.

Table 3. Preparation of Spiking Solutions

ID	Target Concentration (mg/mL)	Source	Source Volume (mL)	Final Volume (mL)
C	0.75	A	3	5
D	0.40	B	2	5
E	0.25	A	2	10
F	0.10	B	1	10

3.3.3 Vehicle/Calibration Standards

One (1) mL from each solution A - F was pipetted into individual 10-mL volumetric flasks. One (1) mL of diluted vehicle and 0.1 mL of IS was added to each volumetric flask. The flasks were diluted to volume with acetone, sealed, and the contents mixed well. This produced single vehicle standards at target concentrations of 0.125, 0.1, 0.075, 0.04, 0.025, and 0.01 mg/mL.

3.3.4 Preparation of Blanks

Vehicle Blank

A single blank was prepared by pipetting 1 mL of diluted vehicle into a 10-mL volumetric flask and diluting to volume with acetone. The flask was sealed and the contents mixed well.

Vehicle Blank with IS

A single blank with IS was prepared by pipetting 1 mL of diluted vehicle and 0.1 mL of IS into a 10-mL volumetric flask and diluting to volume with acetone. The flask was sealed and the contents mixed well.

3.4 Preparation of Formulation Samples For Analysis

3.4.1 Density Determination

For the 0, 20, and 64 mg/mL formulation concentrations, a tared 5-mL volumetric flask was filled to volume with the formulation. The weight of the filled flask was recorded and divided by five to obtain the density of the formulation. For the 200 mg/mL formulation, a tared 1-mL volumetric flask was filled to volume with formulation. The density of this formulation was the weight of the flask contents.

3.4.2 Preparation of Formulation Samples

All formulation samples had a stir bar added to the container. The 200 mg/mL formulations were shaken and vortexed to ensure a uniform sample. The

formulations were stirred for at least 5 minutes prior to use. If necessary, the contents of the vial were transferred to another amber container to allow sufficient stirring before taking samples.

For each formulation with a concentration equal to or less than 100 mg/mL, a 1-mL aliquot was transferred to three previously tared 10-mL volumetric flasks. The weight of the aliquot was recorded. The flasks were diluted to volume with acetone, sealed, and the contents mixed well.

For each formulation with a concentration of 100 mg/mL or greater, a 1-mL aliquot was transferred to three previously tared 25-mL volumetric flasks. The weight of the aliquot was recorded. A 1.5-mL aliquot of corn oil was added to each flask. The flasks were diluted to volume with acetone, sealed, and the contents mixed well.

A 1-mL aliquot of the diluted formulation was transferred to individual 100-mL volumetric flasks. A 1-mL aliquot of IS was added to each flask. The flasks were diluted to volume with acetone, sealed, and the contents mixed well.

3.5 Analysis

Aliquots of each vehicle standard, blank, and sample were transferred into autosampler vials with minimal headspace and the vials were sealed. Single injections were made from each vial using the gas chromatography (GC) instrumental system with flame ionization detection (FID) as shown in Table 4.

Table 4. GC System

Instrument	Agilent 6890 (Santa Clara, CA)
Data System	Thermo Fisher Scientific Atlas, Version 8.2
Column	Restek (Bellefonte, PA), Rtx-5, 30 m × 0.32 mm (ID), 1.0 µm film thickness
Oven Temperature	80°C, hold for 1 minute, increase at 20°C/minute to 200°C, no hold, increase at 10°C/minute to 280°C, hold for 10 minutes
Hydrogen Flow	28 mL/minute
Air Flow Rate	280 mL/minute
Carrier Flow Rate	Helium at 3 mL/minute
Detector Temperature	280°C
Injector Temperature	260°C
Detector Type	FID
Injection Volume/Mode	1 µL/Splitless
Run Time	25 minutes

Representative overlaid chromatograms from a high and low standard, a blank with IS, and a blank are shown in Figure 1.

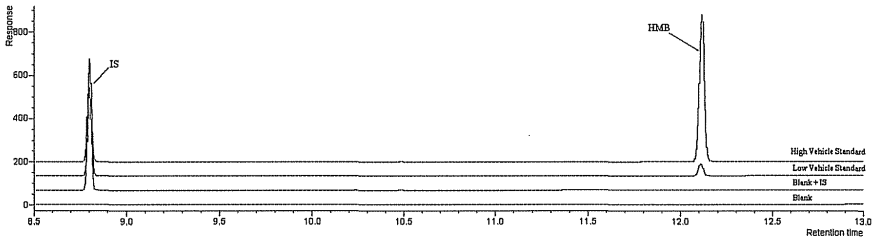


Figure 1. Representative Overlaid Chromatograms

3.6 Calculations

The integration of the HMB and IS peaks done by the chromatography data system was evaluated and manually adjusted, if necessary, to achieve consistent integration. The response ratio of the HMB peak area divided by the IS peak area was calculated. A linear regression equation with 1/x weighting was calculated relating the response ratio of the standards to their nominal concentrations. The determined concentration was calculated for each standard and sample using the regression equation, the response ratio for that standard or sample, the sample weight and density, and any dilution factor for the samples. The relative error (RE) for each standard and sample was calculated by subtracting the target concentration from its determined concentration, dividing the difference by the target concentration, and multiplying the result by 100. The average concentration, average RE, standard deviation, and RSD for each formulation location were calculated using the individual values. The grand average concentration, grand RE, grand standard deviation, and grand RSD for each formulation were calculated using the average concentration for each location.

At least one extra significant figure was carried through all calculations to minimize rounding errors, therefore, the summary statistics presented in the tables may not be exactly reproduced using the rounded input values shown.

3.7 Results

The results of the formulation analyses are shown in Table 5. The 0 mg/mL formulations were all below the limit of quantitation (BLOQ). The standard curve is shown in Figure 2.

Table 5. Corn Oil Homogeneity Formulation Sample Analysis Results

Target Concentration (Sample ID)	Location	Corrected Determined Concentration (mg/mL)	Average Corrected Determined Concentration (mg/mL)	s (mg/mL)	RSD	RE	Avg. RE	Grand Average Concentration (mg/mL)	Grand s (mg/mL)	Grand RSD	Grand RE
20 mg/mL (11-29-1B)	Bottom A	20.3	20.2	0.1	0.5	1.5	0.8	20.2	0.1	0.3	0.8
	Bottom B	20.1				0.5					
	Bottom C	20.1				0.5					
20 mg/mL (11-29-1M)	Middle A	20.2	20.1	0.2	1.0	1.0	0.3				
	Middle B	19.9				-0.5					
	Middle C	20.1				0.5					
20 mg/mL (11-29-1T)	Top A	20.2	20.2	0.1	0.5	1.0	1.0				
	Top B	20.3				1.5					
	Top C	20.1				0.5					
64 mg/mL (11-29-2B)	Bottom A	64.0	64.1	0.2	0.3	0.0	0.1	63.9	0.2	0.3	-0.2
	Bottom B	63.9				-0.2					
	Bottom C	64.3				0.5					
64 mg/mL (11-29-2M)	Middle A	64.2	63.9	0.3	0.5	0.3	-0.2				
	Middle B	63.8				-0.3					
	Middle C	63.6				-0.6					
64 mg/mL (11-29-2T)	Top A	63.8	63.7	0.5	0.8	-0.3	-0.4				
	Top B	64.2				0.3					
	Top C	63.2				-1.3					
200 mg/mL (11-29-3B)	Bottom A	208	208	1	0.5	4.0	4.2	211	2.5	1.2	5.3
	Bottom B	208				4.0					
	Bottom C	209				4.5					
200 mg/mL (11-29-3M)	Middle A	211	211	0	0.0	5.5	5.5				
	Middle B	211				5.5					
	Middle C	211				5.5					
200 mg/mL (11-29-3T)	Top A	214	213	1	0.5	7.0	6.7				
	Top B	213				6.5					
	Top C	213				6.5					

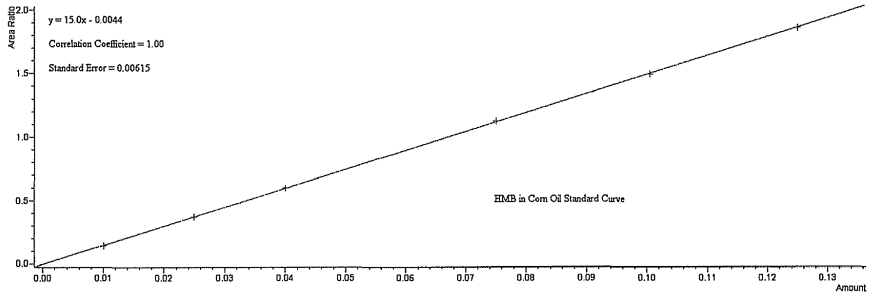


Figure 2. Standard Curve

3.8 Conclusions

The concentrations of all the formulations containing HMB were within 10 percent of target, the NTP acceptance limit. The formulations were also homogeneous. The 0 mg/mL formulation contained no detectable HMB. All other quality criteria stated in the SOP were within acceptance limits.

4.0 ACKNOWLEDGMENTS

[redacted] conducted the analysis. [redacted] wrote the report [redacted] reviewed the analysis raw data for completeness and accuracy.

MRIGlobal

This PDF File is an Exact Copy of the Report
Signature [redacted]
Date: 6-29-11

Analytical Chemistry Services for the NTP
NIEHS Contract No. HHSN273201100001C
MRI Project No.: 110730
NTP ChemTask No.: CHEM10992

Dose Formulation Development Final Report

2-Ethylhexyl p-methoxycinnamate

Dose Formulation Development Study of
2-Ethylhexyl p-methoxycinnamate in Corn Oil

MRI Assignment No.: 2010

June 29, 2011

Prepared by:
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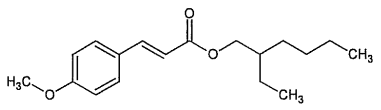
Dose Formulation Development (DFD)

Chemical Information: 2-Ethylhexyl p-methoxycinnamate

CAS No.: 5466-77-3 MRI Assignment No.: 2010 NTP ChemTask No.: CHEM10992 Program Supported: TOX Analysis Dates: 12/7/10 to 1/26/11 Interim Report Dates: 3/29/11 and 3/30/11	Lot No.: A0293319 Supplier: Acros Organics MRI-Assigned Batch No.: 01 Supplier Purity: 99.8% (per C of A); stabilized with 0.09% butylated hydroxytoluene (BHT) Appearance: Clear colorless liquid (per C of A) Storage Condition @ MRI: Ambient (~ 25°C), protected from light Vehicle: Corn oil Vehicle Lot No.: ZT1301 (Spectrum Chemical)

Structure	Mol. Weight	Mol. Formula
	290.39	$C_{18}H_{26}O_3$

Executive Summary

The purpose of this study was to develop and validate a method for the quantitation of 2-ethylhexyl p-methoxycinnamate (EHMC) from a corn oil formulation. The method validation encompassed a formulation range of ~ 1.7 to ~ 268 mg/mL, with a high dose method verification performed at ~ 400 mg/mL. A homogeneity evaluation and 42-day forward storage stability study of an ~ 20 mg/mL and an ~ 200 mg/mL formulation were included with a 3-hour simulated dosing study performed on the ~ 20 mg/mL formulation.

A gas chromatographic method using flame ionization detection (GC/FID) was validated for the analysis of EHMC from the corn oil formulation, using benzyl benzoate as the internal standard. The formulation concentrations ranged from 1.74936 to 268.478 mg/mL, which was equivalent to an analytical concentration range of 75.8924 to 11,647.4 µg/mL EHMC in THF. The method validation proved to be linear (correlation coefficient ≥ 0.999; 1/x weighted linear regression), precise (≤ 4.0% RSD), and accurate (% RE from –7.2% to 4.8%). The mean percent determined/expected ratio (D/E) was 100.0% ± 4.0 (s), with the mean percent recovery for spiked matrix standards relative to solvent standards being 99.7% ± 9.1 (s).

A high dose method verification experiment confirmed that a 404.862 mg/mL formulation can be diluted with blank corn oil into the validated concentration range. The results showed a mean % D/E of 100.0% ± 0.4, 0.4% RSD, n = 3.

The homogeneity evaluations of two formulations, ~ 20 mg/mL and ~ 200 mg/mL EHMC in corn oil, confirmed formulation homogeneity. The % RSD value for the ~ 20 mg/mL dose was 0.6% RSD, n = 9 and 1.4% RSD, n = 9 for the ~ 200 mg/mL dose.

The 42-day stability study was performed on ~ 20 mg/mL and ~ 200 mg/mL EHMC corn oil formulations. The Day 0 mean determined concentration of the low dose was 19.3010 ± 0.1759 mg/mL, 0.9% RSD, n = 9 and 199.496 ± 2.831 mg/mL, 1.4% RSD, n = 9 for the high dose. These formulations were stored under ambient and refrigerated conditions and analyzed on Days 7, 21, 35, and 42. The statistically determined test variability limit (TVL) values for the low dose formulation, at the 95% confidence level, established that any test article percent loss compared to Day 0 which is greater than 1.9% under ambient conditions and greater than 2.7% under refrigerated conditions is statistically significant. The TVL values for the high dose formulation, at the 95% confidence level, established that any test article percent loss compared to Day 0 which is greater than 2.7% under ambient conditions and greater than 2.5% under refrigerated conditions is statistically significant. Using the TVL criteria, it is concluded that formulations of 19.3010 mg/mL EHMC in corn oil and 199.496 mg/mL EHMC in corn oil may be stored for up to 42 days under ambient or refrigerated conditions without the statistically significant loss of EHMC.

The 3-hour simulated dosing study was performed using an ~ 20 mg/mL formulation of EHMC in corn oil. After exposure to air and light, the results indicated a mean determined concentration of 19.3010 ± 0.1759 mg/mL, 0.9 %RSD, n = 9. It was concluded that the formulation can be used for 3 hours during dosing with %D/E values ≥ 95.1%.

In summary, a GC/FID method was validated to cover a formulation range of ~ 1.7 to ~ 268 mg/mL of EHMC in corn oil. The 42-day storage stability study indicated that formulations can be stored under ambient or refrigerated conditions for up to 42 days. The simulated dosing study results indicated that the ~ 20 mg/mL formulation is stable for up to 3 hours, under simulated dosing conditions. In addition, the high dose method verification study results indicated that a high dose formulation may be diluted with corn oil into the validated concentration range.

Quality Assurance Statement

Dose Formulation Development Study of 2-Ethylhexyl p-methoxycinnamate in Corn Oil

NTP ChemTask No. CHEM10992
MRI Project No. 110730
MRI Assignment No. 2010

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

Phase inspected	Date inspected	Date reported
Protocol audit	1/19/11	1/19/11
Protocol Amendment 1 audit	1/19/11	1/19/11
In-Life audit; Day 35 analysis	1/19/11	1/19/11
Protocol Amendment 2 audit	5/18/11	5/18/11
Data audit	5/18/11	5/18/11
Report audit	5/18/11	5/18/11

In addition to the study-specific audits/inspections 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
GC Facility	10/25/10	10/26/10
Laboratory Complex (285 N)	11/11/10	11/12/10

MIDWEST RESEARCH INSTITUTE

[redacted]

Senior Quality Assurance Officer

Approved:

[redacted]

Director, Quality and Regulatory Systems

June 29, 2011

Good Laboratory Practice Compliance Statement

Dose Formulation Development Study of 2-Ethylhexyl p-methoxycinnamate in Corn Oil

NTP ChemTask No. CHEM10992
MRI Project No. 110730
MRI Assignment No. 2010

This dose formulation development study of 2-ethylhexyl p-methoxycinnamate in corn oil 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 MRI Archives.

[redacted]
Study Director

June 29, 2011
Date

Contents

Appendices

Figures

Tables

Dose Formulation Development Study of 2-Ethylhexyl p-methoxycinnamate in Corn Oil

1. Introduction

In anticipation of a gavage study, the purpose of this dose formulation study was to develop and validate a method for the quantitation of 2-ethylhexyl p-methoxycinnamate (EHMC) in corn oil. The method validation was conducted for the quantitation of EHMC in corn oil encompassing a formulation range of ~ 1.7 to ~ 268 mg/mL with a high dose method verification performed at ~ 400 mg/mL. The study also included homogeneity evaluations and 42-day forward storage stability studies of two EHMC formulations in corn oil at ~ 20 mg/mL and ~ 200 mg/mL. A 3-hour simulated dosing study was performed on an ~ 20 mg/mL formulation. This study was initiated on December 9, 2010.

2. Chemical Information

Test Article:	2-Ethylhexyl p-methoxycinnamate (EHMC), stabilized with 0.09% butylated hydroxytoluene (BHT)
Lot No.:	A0293319
MRI-Assigned Batch No.:	01
Supplier:	Acros Organics
Purity:	99.8% (per C of A)
Molecular Formula:	$C_{18}H_{26}O_3$
Molecular Weight:	290.39
CAS No.:	5466-77-3

3. Materials and Equipment

GC system equipped with an Agilent 6890N gas chromatograph with Agilent 7683 autosampler, with FID detector, and TotalChrom data system, Version 6.3.0
Column, DB-5 (Agilent), 30-m × 0.53-mm ID, 1.5-µm film thickness
Benzyl benzoate, 99.0% purity, Sigma, used as internal standard
Tetrahydrofuran (THF), Honeywell, High Purity grade
Corn oil, Spectrum Chemical
Low actinic volumetric glassware, Class A, as needed
Amber GC vials with crimp caps, National Scientific
Amber serum vial, ~ 50 mL, with crimp caps, Kimble Chase

Balances: Mettler Toledo, Model: XS205DU
Mettler Toledo, Model: AG285
Mettler Toledo, Model: XS204

4. Method Development

Initial method development focused on a GC/FID method using a DB-5 column (30 × 0.53 mm ID, and 1.5-µm). Solutions of EHMC, corn oil, and benzyl benzoate were prepared in THF and injected on the GC to evaluate possible interferences. No interferences were observed, so a solvent standard curve of EHMC in THF was prepared from ~ 0.05 to ~ 25 mg/mL to encompass the desired analytical range. The method was optimized for this analytical range by (1) modifying the oven program to achieve optimal peak shape and separation, (2) varying the detector range setting to maximize peak response, and (3) adjusting the injection mode from splitless to split (split ratio of 75:1). Additionally, an appropriate method for dilution of a high dose formulation into the desired analytical range was investigated.

5. Method Validation

Method validation was performed using a GC/FID method for the analysis of EHMC in corn oil to cover a formulation range of 1.74936 to 268.478 mg/mL (or an analytical concentration range of 75.8924 to 11,647.4 µg/mL in THF). The method validation evaluated the linearity, precision, and accuracy of the prepared spiked matrix standards. Percent recovery of EHMC was determined by comparing the response of the spiked matrix standards to the solvent standards at equivalent concentrations. Spiked matrix and solvent standard curves were prepared at six concentrations to cover the analytical range; triplicate preparations at each concentration level were used for the spiked matrix standard curve.

5.1 Preparation of Standards for Method Validation

5.1.1 Internal Standard Solution

An internal standard (IS) solution was prepared by accurately weighing and transferring ~ 4,000 mg of benzyl benzoate into a 100-mL volumetric flask. The contents of the flask were diluted to volume with THF and mixed well; Expected IS concentration was 39.9604 mg/mL.

5.1.2 Stock Solutions

Two stock solutions of EHMC were prepared by accurately weighing and transferring 2,911.85 mg (Stock A) and 1,897.31 mg (Stock B) into individual, 50-mL volumetric flasks, diluting the contents of the flasks to volume with THF, and mixing well. Expected concentrations: Stock A = 58.2370 mg/mL, Stock B = 37.9462 mg/mL.

5.1.3 Intermediate Standard Solutions

Six intermediate standard solutions (IB_1 to IA_6) of EHMC in THF were prepared by transferring aliquots from alternating Stock Solutionss A and B, diluting the contents of the flasks to volume with THF, and mixing well. The preparation scheme is presented in Table 1.

NOTE: IA_6 is EHMC Stock Solution A and IB_5 is EHMC Stock Solution B.

Table 1. Preparation of Intermediate Standard Solutions for Method Validation

Intermediate standard solution	Stock solution	Stock solution aliquot (mL)	Final volume (mL)	Expected concentration in THF (mg/mL)
IB_1	B	1	100	0.379462
IA_2	A	3	50	3.49422
IB_3	B	5	25	7.58924
IA_4	A	8	25	18.6358
IB_5	B	NA	NA	37.9462
IA_6	A	NA	NA	58.2370

5.1.4 Solvent Standards

Six solvent standards (SB_1 to SA_6) were prepared by transferring 5-mL aliquots of the intermediate standard solutions (IB_1 to IA_6, see Section 5.1.3) into individual, 25-mL volumetric flasks containing 2 mL of IS solution. The contents of the flasks were diluted to volume with THF and mixed well. The preparation scheme is presented in Table 2.

Table 2. Preparation of Solvent Standards for Method Validation

Solvent standard	Intermediate standard solution	Intermediate standard solution aliquot (mL)	IS solution (mL)	Final volume (mL)	Expected concentration in THF (µg/mL)
SB_1	IB_1	5	2	25	75.8924
SA_2	IA_2	5	2	25	698.844
SB_3	IB_3	5	2	25	1,517.85
SA_4	IA_4	5	2	25	3,727.17
SB_5	IB_5	5	2	25	7,589.24
SA_6	IA_6	5	2	25	11,647.4

5.1.5 Spiked Matrix Standards

Eighteen, ~ 1-g portions of corn oil were accurately weighed and transferred into individual, 25-mL volumetric flasks; then, 2 mL of IS solution were added to each flask. A 5-mL aliquot of each intermediate standard solution (IB_1 to IA_6, see Section 5.1.3) was transferred into each volumetric flask, with triplicate preparations at each concentration. The contents of the flasks

were diluted to volume with THF and mixed well. The preparation scheme is presented in Table 3.

Table 3. Preparation of Spiked Matrix Standards for Method Validation

Spiked matrix standard	Intermediate standard solution	Intermediate standard solution aliquot (mL)	Corn oil (~ g)	Final volume (mL)[a]	Expected concentration in THF (µg/mL)	Expected concentration in corn oil (mg/mL)[b]
B_{11}, B_{12}, B_{13}	IB_1	5	1	25	75.8924	1.74936
A_{21}, A_{22}, A_{23}	IA_2	5	1	25	698.844	16.1087
B_{31}, B_{32}, B_{33}	IB_3	5	1	25	1,517.85	34.9872
A_{41}, A_{42}, A_{43}	IA_4	5	1	25	3,727.17	85.9131
B_{51}, B_{52}, B_{53}	IB_5	5	1	25	7,589.24	174.936
A_{61}, A_{62}, A_{63}	IA_6	5	1	25	11,647.4	268.478

[a] Contained 2 mL of IS solution.
[b] Density of Corn Oil = 0.92202 g/mL.

5.2 Blank Preparations

5.2.1 Reagent Blank (D_0)

THF was used as a reagent blank.

5.2.2 IS Blank

An IS blank was prepared by transferring 2 mL of IS solution into a 25-mL volumetric flask, diluting the contents of the flask to volume with THF, and mixing well.

5.2.3 Matrix Blanks

Triplicate matrix blanks without IS (C_{01}, C_{02}, and C_{03}) were prepared by transferring accurately weighed ~ 1-g portions of corn oil into individual, 25-mL volumetric flasks, diluting the contents of the flasks to volume with THF, and mixing well.

A matrix blank with IS (C_{04}) was prepared by transferring an accurately weighed ~ 1-g portion of corn oil into a 25-mL volumetric flask, adding 2 mL of IS solution, diluting the contents of the flask to volume with THF, and mixing well.

5.3 Analysis for Method Validation

Aliquots of the solvent standards, spiked matrix standards, and blanks were transferred into individual autosampler vials and analyzed using the GC system and parameters described in Table 4.

Table 4. GC Conditions for Method Validation

Gas Chromatograph:	Agilent 6890N gas chromatograph with Agilent 7683 autosampler
Column:	DB-5 (Agilent), 30 m × 0.53 mm ID,1.5-µm film thickness
Liner Type:	Dual Tapered with glass wool
Injector Temperature:	280°C
Injector Mode:	Split, Split Ratio 75:1
Detector:	Flame Ionization Detector (FID)
Detector Temperature:	300°C
Detector Range:	1
Carrier Gas:	Helium
Carrier Gas Flow Rate:	~ 10.0 mL/min
Hydrogen Flow:	~ 30 mL/min
Air Flow:	~ 300 mL/min
Make-up Gas:	Nitrogen
Make-up Gas Flow Rate:	~ 25 mL/min
Injection Volume:	1 µL
Oven Program:	85°C (1-min hold), 15.0°C/min to 255°C (5-min hold) 15°C/min to 300°C (5-min hold)
Run Time:	25 min
Data System:	TotalChrom, Version 6.3.0
Retention Times:	EHMC: ~ 14.6 min Benzyl benzoate (IS): ~ 10.6 min

5.4 Calculations for Method Validation

1. A peak area ratio (PAR) for EHMC was calculated as follows:

$$\text{PAR} = \frac{\text{Peak Area (EHMC)}}{\text{Peak Area (IS)}}$$

2. The slope, y-intercept, and correlation coefficient were calculated from a weighted (1/x) linear regression analysis of the spiked matrix standard curve by relating the PAR of each spiked matrix standard with its corresponding expected concentration (µg/mL in THF).

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

$$\text{Determined Concentration (mg/mL)} = \frac{[\text{PAR} - (\text{y-intercept})]}{\text{slope}} \times \frac{\text{d}}{\sim 1\text{ g corn oil}} \times \text{DF} \times \frac{1\text{ mg}}{1{,}000\ \mu\text{g}}$$

where: d = density of corn oil (g/mL) = 0.92202 g/mL
DF = dilution factor (25 mL)

4. The slope, y-intercept, and correlation coefficient were calculated from a weighted (1/x) linear regression analysis of the solvent standard curve by relating the PAR of each solvent standard with its corresponding expected concentration (µg/mL in THF).

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

$$\text{Determined Concentration } (\mu g/mL) = \frac{[\text{PAR} - (\text{y} - \text{intercept})]}{\text{slope}}$$

6. Method precision, expressed as percent relative standard deviation (% RSD), was calculated from the mean PAR for the triplicate preparations at all the concentrations of spiked matrix standards.

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

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

8. Method accuracy, expressed as percent relative error (% RE), was calculated as follows:

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

9. For each spiked matrix standard, the percent recovery was calculated relative to the corresponding solvent standard as follows:

$$\%\ \text{Recovery} = \left(\frac{\text{PAR (Spiked matrix stds)}}{\text{PAR (Solvent stds)}} \right) \times 100$$

10. The limit of detection (LOD) and limit of quantitation (LOQ) were determined based on the standard deviation (s) of the lowest spiked matrix standard expressed as the determined concentration and calculated as follows:

$$LOD = 3 \times s$$

$$LOQ = 10 \times s$$

11. The experimental limit of quantitation (ELOQ) was defined as the lowest mean determined concentration (mg/mL in corn oil) of spiked matrix standard with a % RE $\leq \pm 10\%$ and a % RSD $\leq 10\%$.

12. System suitability parameters were calculated for system precision, peak tailing (T), theoretical plates (N), and resolution (R) according to USP guidelines.[1] System precision was calculated using the mean PAR determined 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.

13. Sample mean ($\bar{x}$), standard deviation (s), and % RSD were calculated using commonly accepted techniques.

[1] *United States Pharmacopeia* [621] Chromatography, (2008), official from May 1, 2008, 31st Edition, Volume 1, pp. 232–243.

5.5 Results of Method Validation

A method validation was performed for the analysis of EHMC in corn oil that covered a formulation range of 1.74936 to 268.478 mg/mL in corn oil (or an analytical concentration range of 75.8924 to 11,647.4 µg/mL in THF). The spiked matrix standard curve proved to be linear ($r \geq 0.999$), precise (% RSD ≤ 4.0), and accurate (% RE from –7.2% to 4.8%). The mean percent D/E for the spiked matrix standards (n = 17; spiked matrix standard B_{33} was rejected by the Q-test at the 90% confidence level) was 100.0% ± 4.0 (s). The overall percent recovery for the spiked matrix standards relative to solvent standards at the same concentration was 99.7% ± 9.1 (s) (n = 17; spiked matrix standard B_{33} was rejected by the Q-test at the 90% confidence level). The estimated LOD for the method was 0.05958 mg/mL, the estimated LOQ was 0.19860 mg/mL, and the ELOQ was 1.64389 mg/mL [% RSD = 1.2% (n = 3)]. The data from the method validation are presented in Table 10 and are graphically represented in Figure 1. A representative chromatogram is presented in Figure 2. System suitability data calculated from the method validation are presented in Table 5. Method criteria were established based on the system suitability data.

Table 5. System Suitability Results for Method Validation

System precision	Theoretical plates	Tailing factor	Resolution
Analytical results: % RSD = 1.2 (n = 6)	EHMC = 298,295.75	EHMC = 0.984	49.507
	IS = 634,948.03	IS = 0.988	
Method criteria: % RSD ≤ 2.0	EHMC ≥ 200,000	0.6 ≤ T ≤ 1.2	≥ 40
	IS ≥ 400,000	0.6 ≤ T ≤ 1.2	

6. 42-Day Stability Study of EHMC in Corn Oil (Forward Method)

A 42-day stability study was conducted on an ~ 20 mg/mL and an ~ 200 mg/mL formulation of EHMC in corn oil. The formulations were evaluated for homogeneity immediately after preparation. The formulations were stored under ambient (~ 25°C) and refrigerated (~ 5°C) conditions and analyzed on Days 7, 21, 35, and 42.

NOTE: Per the NTP Contracting Officer's Technical Representative (COTR), the Day 14 stability time point specified in the Statement of Work (SOW) was not included in this study.

6.1 Formulations of EHMC in Corn Oil

6.1.1 20 mg/mL Formulation of EHMC in Corn Oil

6.1.1.1 Preparation and Storage of ~ 20 mg/mL EHMC in Corn Oil—Day 0

A dose formulation was prepared by accurately weighing and transferring ~ 20 g of EHMC into a 1-L volumetric flask followed by adding 200 mL of corn oil. The contents of the flask were mixed well. The process of adding 200 mL of corn oil and mixing well was repeated until the contents of the flask were diluted to volume with corn oil. The formulation was initially used for homogeneity determination and 3-hour simulated dosing analysis. The remaining formulation was aliquoted into individual, amber glass containers and stored under ambient (~ 25°C) and refrigerated (~ 5°C) conditions for the 42-day stability analyses. The density of EHMC was 1.0070 g/cm^3 per MSDS. Expected concentration of the formulation was 19.9542 mg/mL.

NOTE: The homogeneity determination for the ~ 20 mg/mL dose formulation on Day 0 did not pass criteria for the relative standard deviation (± 5%) due to insufficient mixing. Upon further examination, it was determined that mixing with a magnetic stir bar is required to ensure formulation homogeneity. Homogeneity was repeated on Day 7 of the stability study using a freshly prepared dose formulation of ~ 20 mg/mL of EHMC in corn oil. The determined concentration of the 3-hour simulated dose (described in Section 7) was used as the Day 0 value for the stability study.

6.1.1.2 Preparation of ~ 20 mg/mL EHMC in Corn Oil—Day 7

A dose formulation was prepared as described in Section 6.1.1.1, followed by mixing with a magnetic stir bar for ≥ 5 minutes. The formulation was used for homogeneity determination only. Expected concentration of the formulation was 20.6200 mg/mL.

6.1.2 Density Determinations of the ~ 20 mg/mL Formulations

6.1.2.1 Density Determination of the ~ 20 mg/mL Formulation—Day 0

The density of the ~ 20 mg/mL formulation was determined by transferring an aliquot of the formulation into three, individual, pre-weighed 10-mL volumetric flasks. The weights of the filled flasks were recorded and the densities and average density were calculated using commonly accepted methods. The average density (n = 3) was determined to be 0.92343 g/mL.

6.1.2.2 Density Determination of the ~ 20 mg/mL Formulation—Day 7

The density of the ~ 20 mg/mL formulation was determined as described in Section 6.1.2.1. The average density (n = 3) was determined to be 0.92061 g/mL.

6.1.3 200 mg/mL Formulation of EHMC in Corn Oil

6.1.3.1 Preparation and Storage of ~ 200 mg/mL EHMC in Corn Oil

A dose formulation was prepared by accurately weighing and transferring 200.2383 g of EHMC into a 1-L volumetric flask followed by adding 200 mL of corn oil. The contents of the flask were mixed well. The process of adding 200 mL of corn oil and mixing well was repeated until the contents of the flask were diluted to volume with corn oil. The formulation was initially used for homogeneity determination. The remaining formulation was aliquoted into individual, amber glass containers and stored under ambient (~ 25°C) and refrigerated (~ 5°C) conditions for the 42-day stability analyses. The density of EHMC was 1.0070 g/cm^3 per MSDS. Expected concentration of the formulation was 200.238 mg/mL.

6.1.4 Density Determination of the ~ 200 mg/mL Formulation

The density of the ~ 200 mg/mL formulation was determined by transferring an aliquot of the formulation into three, individual, pre-weighed 10-mL volumetric flasks. The weights of the filled flasks were recorded and the densities and average density were calculated using commonly accepted methods. The average density (n = 3) was determined to be 0.93751 g/mL.

6.2 Homogeneity Evaluation and Stability Study Analyses

The following procedure was followed for the homogeneity evaluation (Day 0) and for each stability study time-point (Day 7, 21, 35, and 42).

6.2.1 Preparation of Standards

6.2.1.1 Internal Standard Solution

An IS solution was prepared as described in Section 5.1.1; Expected IS concentration was ~ 40 mg/mL.

6.2.1.2 Stock Solutions

Two stock solutions (A and B) of EHMC were prepared by accurately weighing and transferring ~ 2,900 mg and ~ 1,900 mg into individual, 50-mL volumetric flasks, diluting the contents of the flasks to volume with THF, and mixing well. Expected concentrations: Stock A = ~ 58 mg/mL, Stock B = ~ 38 mg/mL.

6.2.1.3 Intermediate Standard Solutions

Six intermediate standard solutions (IB_1 to IA_6) of EHMC in THF were prepared by transferring aliquots from alternating Stock Solutions A and B, diluting the contents of the flasks to volume with THF, and mixing well. The preparation scheme is presented in Table 6.

NOTE: IA_6 is EHMC Stock Solution A and IB_5 is EHMC Stock Solution B.

Table 6. Preparation of Intermediate Standard Solutions for Homogeneity Evaluations and Stability Study Analyses

Intermediate standard solution	Stock solution	Stock solution aliquot (mL)	Final volume (mL)	Expected concentration in THF (~ mg/mL)
IB_1	B	1	100	0.38
IA_2	A	3	50	3.48
IB_3	B	5	25	7.60
IA_4	A	8	25	18.56
IB_5	B	NA	NA	38.00
IA_6	A	NA	NA	58.00

6.2.1.4 Solvent Standards

Six solvent standards (SB_1 to SA_6) were prepared by transferring 5-mL aliquots of the intermediate standard solutions (IB_1 to IA_6, see Section 6.2.1.3) into individual, 25-mL volumetric flasks containing 2 mL of IS solution. The contents of the flasks were diluted to volume with THF and mixed well. The preparation scheme is presented in Table 7.

Table 7. Preparation of Solvent Standards for Homogeneity Evaluations and Stability Study Analyses

Solvent standard	Intermediate standard solution	Intermediate standard solution aliquot (mL)	IS solution (mL)	Final volume (mL)	Expected concentration in THF (~ µg/mL)
SB_1	IB_1	5	2	25	76
SA_2	IA_2	5	2	25	696
SB_3	IB_3	5	2	25	1,520
SA_4	IA_4	5	2	25	3,712
SB_5	IB_5	5	2	25	7,600
SA_6	IA_6	5	2	25	11,600

6.2.1.5 Spiked Matrix Standards

Six spiked matrix standards (B_1 to A_6) were prepared by transferring accurately weighed ~ 1-g portions of corn oil into individual, 25-mL volumetric flasks and adding 2 mL of IS solution to each flask; then, a 5-mL aliquot of each intermediate standard solution (IB_1 to IA_6,

see Section 6.2.1.3) was transferred into each volumetric flask. The contents of the flasks were diluted to volume with THF and mixed well. The preparation scheme is presented in Table 8.

Table 8. Preparation of Spiked Matrix Standards for Homogeneity Evaluations and Stability Study Analyses

Spiked matrix standard	Intermediate standard solution	Intermediate standard solution aliquot (mL)	Corn oil (~ g)	Final volume (mL)[a]	Expected concentration in THF (~ µg/mL)	Expected concentration in corn oil (~ mg/mL)[b]
B_1	IB_1	5	1	25	76	1.75
A_2	IA_2	5	1	25	696	16.04
B_3	IB_3	5	1	25	1,520	35.04
A_4	IA_4	5	1	25	3,712	85.56
B_5	IB_5	5	1	25	7,600	175.18
A_6	IA_6	5	1	25	11,600	267.39

[a] Contained 2 mL of IS solution.
[b] Density of Corn Oil = 0.92202 g/mL.

6.2.2 Blank Preparations

6.2.2.1 Reagent Blank (D_0)

THF was used as a reagent blank.

6.2.2.2 IS Blank

An IS blank was prepared as described in Section 5.2.2.

6.2.2.3 Matrix Blanks

Two matrix blanks (C_{01} and C_{04}) were prepared as described in Section 5.2.3.

NOTE: The matrix blank containing IS solution (C_{04}) was renamed C_{02}.

6.2.3 Homogeneity Samples

Directly after preparation of the ~ 20 mg/mL formulation (prepared on Day 7) and ~ 200-mg/mL formulation (Section 6.1), triplicate ~ 1-g portions from the top, middle, and bottom locations (n = 9, for each formulation) were accurately weighed into individual, 25-mL volumetric flasks. A 2-mL aliquot of IS solution was added to each flask. The contents of the flasks were diluted to volume with THF and mixed well. Expected concentrations: Low dose formulation = 20.6200 mg/mL, High dose formulation = 200.238 mg/mL.

6.2.4 Stability Study Samples

On Days 7, 21, 35, and 42 samples were removed from the ambient (~ 25°C) and refrigerated (~ 5°C) storage conditions. The refrigerated samples were allowed to equilibrate to ambient temperature. While the formulation was stirred continuously, triplicate ~ 1-g portions of the formulation were accurately weighed into individual 25-mL volumetric flasks. A 2-mL aliquot of IS solution was added to each flask. The contents of the flasks were diluted to volume with THF and mixed well.

6.3 Analyses for 42-Day Stability Study

Aliquots of the solvent standards, spiked matrix standards, homogeneity samples, stability samples, and blanks were transferred into individual autosampler vials and analyzed using the GC system and parameters described in Table 4 (Section 5.3).

6.4 Calculations

1. A peak area ratio (PAR) for EHMC was calculated as follows:

$$PAR = \frac{\text{Peak Area (EHMC)}}{\text{Peak Area (IS)}}$$

2. The slope, y-intercept, and correlation coefficient were calculated from a weighted (1/x) linear regression analysis of the spiked matrix standard curve by relating the PAR of each spiked matrix standard with its corresponding expected concentration (µg/mL in THF).
3. Using the slope and y-intercept determined from the spiked matrix standard curve and the PAR for each spiked matrix standard, the determined concentration in corn oil of each spiked matrix standard was calculated using the following equation:

$$\text{Determined Concentration (mg/mL)} = \frac{[\text{PAR} - (\text{y-intercept})]}{\text{slope}} \times \frac{\text{d}}{\sim 1\text{ g corn oil}} \times \text{DF} \times \frac{1\text{ mg}}{1{,}000\ \mu\text{g}}$$

where: d = density of corn oil (g/mL) = 0.92202 g/mL
DF = dilution factor (25 mL)

4. The slope, y-intercept, and correlation coefficient were calculated from a weighted (1/x) linear regression analysis of the solvent standard curve by relating the PAR of each solvent standard with its corresponding expected concentration (µg/mL in THF).
5. Using the slope and y-intercept determined from the solvent standard curve and the PAR for each solvent standard, the determined concentration of each solvent standard was calculated using the following equation:

$$\text{Determined Concentration (µg/mL)} = \frac{[\text{PAR} - (\text{y} - \text{intercept})]}{\text{slope}}$$

6. Method accuracy, expressed as percent relative error (% RE), was calculated as follows:

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

7. Using the slope and y-intercept determined from the spiked matrix standard curve prepared on the day of analysis, the determined formulation concentration of each homogeneity and stability sample was calculated using the following equation:

$$D = \frac{[\text{PAR (sample)} - (\text{y - intercept})]}{\text{slope}} \times \frac{\text{Density (g/mL) of sample}}{\text{Sample Weight (g)}} \times \text{DF} \times \frac{1 \text{ mg}}{1{,}000 \text{ µg}}$$

where: D = determined concentration (mg/mL)
DF = dilution factor (25 mL)

8. For each spiked matrix standard, the percent recovery was calculated relative to the corresponding solvent standard as follows:

$$\% \text{ Recovery} = \left(\frac{\text{PAR (Spiked matrix stds)}}{\text{PAR (Solvent stds)}} \right) \times 100$$

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

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

10. The mean ($\bar{x}$), standard deviation (s), and percent relative standard deviation (% RSD) of the determined concentration of each triplicate stability sample and homogeneity sample (top, middle, and bottom) were calculated using commonly accepted techniques.

11. The calculated determined concentration (D) was compared to the mean Day 0 determined concentration (Z) as follows:

$$\% \text{ D/Z} = \frac{D}{Z} \times 100$$

12. System suitability parameters were calculated for system precision, peak tailing (T), theoretical plates (N), and resolution (R) according to USP guidelines.[1] System precision was calculated using the mean PAR determined 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.

6.5 Results of Homogeneity Evaluation/Stability Study

A 42-day stability study was performed at ~ 20 mg/mL and ~ 200 mg/mL EHMC in corn oil. The formulations were evaluated for homogeneity on Day 0. However, the homogeneity determination for the ~ 20 mg/mL dose formulation did not meet criteria for the relative standard deviation (± 5%) due to insufficient mixing. Evaluation of the results from the 3-hour simulated

dosing study (see Section 7) performed on Day 0 showed that homogeneity criteria were met. Thus, the mean determined concentration from the 3-hour simulated dosing study (19.3010 mg/mL, Section 7.3) was used as the Day 0 value for the stability study. In order to show that a homogeneous, ~ 20 mg/mL dose formulation could be prepared, the homogeneity experiment was repeated on Day 7 of the stability study. The homogeneity results indicated the following:

- **~ 20 mg/mL Formulation:** homogeneous (0.6% RSD), with a mean determined concentration of 20.3227 ± 0.1131 mg/mL.
- **~ 200 mg/mL Formulation:** homogeneous (1.4% RSD), with a mean determined concentration of 199.496 ± 2.831 mg/mL.

The formulations were stored under ambient (~ 25°C) and refrigerated (~ 5°C) conditions and analyzed on Days 7, 21, 35, and 42. When compared to the Day 0 determined concentrations (19.3010 mg/mL and 199.496 mg/mL, respectively), the results of the 42-day stability study indicated:

- **~ 20 mg/mL Formulation:**
  - Loss of ≤ 1.1% under ambient conditions
  - Loss of ≤ 0.4% under refrigerated conditions
- **~ 200 mg/mL Formulation:**
  - Loss of ≤ 1.2% under ambient conditions
  - Loss of ≤ 1.8% under refrigerated conditions

The statistically determined test variability limit (TVL) values for the formulations, at the 95% confidence level, established that any test article percent loss compared to Day 0 which meets the following criteria is statistically significant:

- **~ 20 mg/mL Formulation:**
  - Percent loss greater than 1.9% under ambient conditions
  - Percent loss greater than 2.7% under refrigerated conditions
- **~ 200 mg/mL Formulation:**
  - Percent loss greater than 2.7% under ambient conditions
  - Percent loss greater than 2.5% under refrigerated conditions

Using the TVL criteria, it is concluded that formulations of 19.3010 mg/mL EHMC in corn oil and 199.496 mg/mL EHMC in corn oil may be stored for up to 42 days under ambient or refrigerated conditions without the statistically significant loss of EHMC.

System suitability parameters were monitored over the 42-day stability study and method criteria were met on each day of analysis. The results are presented in Table 11. A summary of the spiked matrix standard curve parameters determined during the 42-day stability study are presented in Table 12. The results of the homogeneity study are presented in Tables 13 and 14.

The results of the 3-hour simulated dosing study (see Section 7), which were used to determine the Day 0 value for the ~ 20 mg/mL formulation for the stability study, are presented in Table 15. The tabulated results from the 42-day stability study are presented in Tables 16 to 19 and are graphically represented in Figures 3 and 4.

7. 3-Hour Simulated Dosing Study of ~ 20 mg/mL EHMC in Corn Oil

On Day 0, a portion (~ 50 mL) of the prepared ~ 20 mg/mL formulation (see Section 6.1) was transferred into a clear, 50-mL serum vial and exposed to light and air. To simulate dosing conditions, 1-mL aliquots of the formulation were removed at 15-minute intervals and discarded. After 3 hours, triplicate ~ 1-g portions from each of the top, middle, and bottom locations (n = 9) of the formulation were accurately weighed into individual, 25-mL volumetric flasks. A 2-mL aliquot of IS solution was added to each flask. The contents of the flasks were diluted to volume with THF and mixed well.

7.1 Analysis for 3-Hour Simulated Dosing Study

Aliquots of the simulated dosing samples were transferred into individual autosampler vials and analyzed using the GC system and parameters described in Table 4 (Section 5.3).

7.2 Calculations

See Section 6.4 for calculations.

7.3 Results of 3-Hour Simulated Dosing Study

A 3-hour simulated dosing study was performed at ~ 20 mg/mL EHMC in corn oil in which the formulation was exposed to air and light. The results of the 3-hour simulated dosing study indicated a mean determined concentration of 19.3010 ± 0.1759 mg/mL, 0.9% RSD, n = 9. The mean determined concentration for the 3-hour simulated dosing study was used as the Day 0 value for the stability study, since the homogeneity determination for the ~ 20 mg/mL dose formulation on Day 0 did not meet criteria for the relative standard deviation (± 5%) (Section 6.5). It was concluded that the formulation can be used for 3 hours during dosing with % D/E values ≥ 95.1%. The results of the 3-hour simulated dosing study are presented in Table 15.

8. High Dose Method Verification at ~ 400 mg/mL EHMC in Corn Oil

On Day 7 of the stability study, a high dose method verification experiment was performed to show that a high dose formulation, ~ 400 mg/mL, could be diluted into the concentration range of the validated curve.

8.1 High Dose Formulation Preparation

8.1.1 Preparation of ~ 400 mg/mL EHMC in Corn Oil

A dose formulation was prepared by transferring 40.4847 g of EHMC into a tared, 100-mL volumetric flask, followed by adding 20 mL of corn oil. The contents of the flask were mixed well. The contents of the flask were then diluted to volume with corn oil and mixed by inversion. Finally, the contents of the flask were mixed using a magnetic stir bar for ≥ 5 minutes. Expected concentration of formulation: 404.847 mg/mL.

8.1.2 Density Determination of the ~ 400-mg/mL Formulation

The density of the ~ 400-mg/mL formulation was determined by transferring an aliquot of the formulation into three, individual, pre-weighed 10-mL volumetric flasks. The weights of the filled flasks were recorded and the densities and average density were calculated using commonly accepted methods. The average density (n = 3) was determined to be 0.95623 g/mL.

8.2 High Dose Formulation Dilution

8.2.1 Preparation of ~ 40 mg/mL EHMC Formulation in Corn Oil

The high dose formulation was diluted by transferring triplicate, ~ 5-g aliquots into individual, 50-mL volumetric flasks labeled (xHD1, xHD2, and xHD3). The contents of the flasks were diluted to volume with corn oil and mixed well. Expected concentrations of diluted formulations: ~ 40 mg/mL.

8.2.2 Density Determination of the ~ 40-mg/mL Formulations

The densities of the ~ 40 mg/mL formulations were determined by transferring an aliquot of each of the three formulations into individual, pre-weighed 10-mL volumetric flasks. The weights of the filled flasks were recorded and the densities and average density were calculated using commonly accepted methods. The average density (n = 3) was determined to be 0.92384 g/mL.

8.3 Preparation of High Dose Method Verification Samples

After preparation and dilution (Sections 8.1 and 8.2), the three diluted high dose formulations were prepared for analysis by accurately weighing and transferring individual, ~ 1-g portions into three, 25-mL volumetric flasks labeled (HD1, HD2, and HD3). A 2-mL aliquot of IS solution was added to each flask. The contents of the flasks were diluted to volume with THF and mixed well. The preparation scheme is presented in Table 9.

Table 9. Preparation of High Dose Method Verification Samples for Analysis

High dose method verification samples	Expected high dose formulation concentration (mg/mL)	High dose formulation sample (g)	Initial dilution in corn oil (mL)	Aliquot of initial dilution (g)	Final dilution (mL)[a]	Expected concentration in THF (µg/mL)[b, c]
HD1	404.847	5.01184	50	1.05101	25	1931.19
HD2	404.847	5.01792	50	1.01080	25	1859.56
HD3	404.847	5.01678	50	1.02328	25	1882.09

[a] Contained 2 mL of IS solution.
[b] Density of high dose formulation = 0.95623 g/mL.
[c] Density of diluted high dose formulation = 0.92384 g/mL.

8.4 Analysis for High Dose Method Verification

Aliquots of the high dose method verification samples were transferred into individual autosampler vials and analyzed using the GC system and parameters described in Table 4 (Section 5.3).

8.5 Calculations for High Dose Method Verification

1. A peak area ratio (PAR) for EHMC was calculated as follows:

$$PAR = \frac{\text{Peak Area (EHMC)}}{\text{Peak Area (IS)}}$$

2. The slope, y-intercept, and correlation coefficient were calculated from a weighted (1/x) linear regression analysis of the spiked matrix standard curve by relating the PAR of each spiked matrix standard with its corresponding expected concentration (µg/mL in THF).

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

$$\text{Determined Concentration (mg/mL)} = \frac{[\text{PAR} - (\text{y-intercept})]}{\text{slope}} \times \frac{d}{\sim 1 \text{ g corn oil}} \times DF \times \frac{1 \text{ mg}}{1{,}000 \text{ µg}}$$

where: d = density of corn oil (g/mL) = 0.92202 g/mL
DF = dilution factor (25 mL)

4. The slope, y-intercept, and correlation coefficient were calculated from a weighted (1/x) linear regression analysis of the solvent standard curve by relating the PAR of each solvent standard with its corresponding expected concentration (µg/mL in THF).
5. Using the slope and y-intercept determined from the solvent standard curve and the PAR for each solvent standard, the determined concentration of each solvent standard was calculated using the following equation:

$$\text{Determined Concentration (µg/mL)} = \frac{[\text{PAR - (y - intercept)}]}{\text{slope}}$$

6. Method accuracy, expressed as percent relative error (% RE), was calculated as follows:

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

7. Using the slope and y-intercept determined from the spiked matrix standard curve, the determined formulation concentration of each high dose method verification sample was calculated using the following equation:

$$\text{D} = \frac{[\text{PAR (sample) - (y - intercept)}]}{\text{slope}} \times \text{DF1} \times \text{DF2} \times \frac{1\text{ mg}}{1{,}000\ \text{µg}}$$

where: D = determined concentration (mg/mL)
DF1 = high dose formulation dilution factor
DF2 = diluted high dose formulation dilution factor

$$\text{DF1} = \frac{\text{Density (g/mL) of High Dose}}{\text{High Dose Sample Weight (g)}} \times 50\text{ mL}$$

$$\text{DF2} = \frac{\text{Density (g/mL) of Diluted High Dose}}{\text{Diluted High Dose Sample Weight (g)}} \times 25\text{ mL}$$

8. For each spiked matrix standard, the percent recovery was calculated relative to the corresponding solvent standard as follows:

$$\%\,\text{Recovery} = \left(\frac{\text{PAR (Spiked matrix stds)}}{\text{PAR (Solvent stds)}}\right) \times 100$$

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

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

10. The mean ($\bar{x}$), standard deviation (s), and percent relative standard deviation (% RSD) of the determined concentration of each triplicate high dose method verification sample were calculated using commonly accepted techniques.
11. System suitability parameters were calculated for system precision, peak tailing (T), theoretical plates (N), and resolution (R) according to USP guidelines.[1] System precision was calculated using the mean PAR determined 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.

8.6 Results of High Dose Method Verification

A high dose method verification experiment was performed to show that a formulation of ~ 400 mg/mL EHMC in corn oil could be diluted into the concentration range of the validated curve. The results indicated that the concentration of a high dose formulation could be accurately determined by diluting with blank corn oil. The mean determined concentration of the formulation was 404.862 ± 1.561 mg/mL, 0.4% RSD, n = 3. The % D/E values ranged from 99.6% to 100.4%. The results of the high dose method verification are presented in Table 20.

9. Conclusions

The purpose of this dose formulation study was to develop and validate a method for the quantitation of 2-ethylhexyl p-methoxycinnamate (EHMC) in corn oil. The method validation was conducted for the quantitation of EHMC in corn oil encompassing a formulation range of ~ 1.7 to ~ 268 mg/mL with a high dose method verification performed at ~ 400 mg/mL. The study also included homogeneity evaluations and 42-day forward storage stability studies of two EHMC formulations in corn oil at ~ 20 mg/mL and ~ 200 mg/mL. A 3-hour simulated dosing study was performed on an ~ 20 mg/mL formulation.

A method validation using GC/FID was performed for the analysis of EHMC in corn oil to cover a formulation concentration range of 1.74936 to 268.478 mg/mL (analytical concentration range: 71.3167 to 11,601.5 µg/mL EHMC in THF). The method validation proved to be linear (correlation coefficient ≥ 0.999), precise (≤ 4.0% RSD), and accurate (% RE from –7.2% to 4.8%). The mean percent D/E for the spiked matrix standards was 100.0% ± 4.0 (s). The mean percent recovery for spiked matrix standards relative to solvent standards at the same concentration was 99.7% ± 9.1 (s).

The high dose method verification experiment confirmed that an ~ 400 mg/mL EHMC in corn oil formulation can be diluted with blank corn oil into the validated concentration range. The results showed a mean % D/E of 100.0% ± 0.4, 0.4% RSD, n = 3.

The homogeneity evaluations of two formulations, ~ 20 mg/mL and ~ 200 mg/mL EHMC in corn oil, confirmed that both formulations were homogeneous. The % RSD value for an ~ 20-mg/mL dose was 0.6% RSD, n = 9 and 1.4% RSD, n = 9 for an ~ 200-mg/mL dose. The homogeneity evaluations were performed on Day 0, however, the homogeneity determination for the ~ 20 mg/mL dose formulation did not meet criteria for the relative standard deviation (± 5%) due to insufficient mixing. In order to show that a homogeneous, ~ 20 mg/mL dose formulation could be prepared, the homogeneity evaluation of an ~ 20 mg/mL formulation was repeated on Day 7 of the stability study, incorporating the use of a magnetic stir bar to facilitate mixing. The results of this evaluation met criteria. Thus, it was shown that the use of a magnetic stir bar is needed during preparation of EHMC formulations in corn oil to ensure formulation homogeneity.

The 42-day stability study was performed using dose formulations of ~ 20 mg/mL and ~ 200 mg/mL EHMC in corn oil. The Day 0 mean determined concentration of the low dose was 19.3010 ± 0.1759 mg/mL, 0.9% RSD, n = 9 and 199.496 ± 2.831 mg/mL, 1.4% RSD, n = 9 for the high dose. These formulations were stored under ambient and refrigerated conditions and analyzed on Days 7, 21, 35, and 42. When compared to the Day 0 determined concentrations, the results of the 42-day stability study indicated losses of ≤ 1.1% under ambient conditions and ≤ 0.4% under refrigerated conditions for the low dose and losses of ≤ 1.2% under ambient conditions and ≤ 1.8% under refrigerated conditions for the high dose. The statistically determined test variability limit (TVL) values for the low dose formulation, at the 95% confidence level, established that any test article percent loss compared to Day 0 which is greater than 1.9% under ambient conditions and greater than 2.7% under refrigerated conditions is statistically significant. The TVL values for the high dose formulation, at the 95% confidence level, established that any test article percent loss compared to Day 0 which is greater than 2.7% under ambient conditions and greater than 2.5% under refrigerated conditions is statistically significant. Using the TVL criteria, it was concluded that the low dose formulation can be stored for 42 days under ambient conditions with recoveries ≥ 98.9% or 42 days under refrigerated conditions with recoveries ≥ 99.6% and that the high dose formulation can be stored for 42 days under ambient conditions with recoveries ≥ 98.8% or 42 days under refrigerated conditions with recoveries ≥ 98.2%.

A 3-hour simulated dosing study was performed at ~ 20 mg/mL EHMC in corn oil in which the formulation was exposed to air and light. The results of the 3-hour simulated dosing study indicated a mean determined concentration of 19.3010 ± 0.1759 mg/mL, 0.9 %RSD, n = 9. The mean determined concentration for the 3-hour simulated dosing study was used as the Day 0 value for the 42-day stability study, since the homogeneity determination for the ~ 20 mg/mL dose formulation on Day 0 did not meet criteria for the relative standard deviation (± 5%). It was concluded that the formulation can be used for 3 hours during dosing with mean % D/E values ≥ 95.8%.

In summary, a GC/FID method was validated to cover a formulation range of ~ 1.7 to ~ 268 mg/mL and a high dose formulation (~ 400 mg/mL) may be diluted with corn oil into the validated concentration range. The results of the storage stability study indicated that formulations of EHMC in corn oil can be stored under ambient or refrigerated conditions for up to 42 days. The simulated dosing study results indicated that the ~ 20-mg/mL formulation is stable for up to 3 hours under simulated dosing conditions.

10. Contributors

Personnel contributing to this study were [redacted].

Table 10. Results for Method Validation of EHMC in Corn Oil

Weighted (1/x) Linear regression parameters							
			Spiked matrix standard data			Solvent standard data	
Correlation coefficient			0.999749			0.999743	
Slope			0.000293310			0.000318926	
Y–intercept			0.00239075			–0.00644819	
Spiked matrix standard	Expected (E) concentration (mg/mL)	Spiked matrix standard PAR[a]	Determined (D) concentration (mg/mL)[b]	% D/E[c]	% Relative Error[d]	Solvent standard PAR[a]	% Recovery[e]
B_{11}	1.74936	0.023537	1.66183	95.0	–5.0	0.020465	115.0
B_{12}	1.74936	0.023352	1.64729	94.2	–5.8	f	114.1
B_{13}	1.74936	0.023037	1.62254	92.8	–7.2	f	112.6
			$\bar{x}$ = 1.64389 ± 0.01986 (s)				
			1.2% RSD				
A_{21}	16.1087	0.215249	16.7280	103.8	3.8	0.204051	105.5
A_{22}	16.1087	0.216817	16.8512	104.6	4.6	f	106.3
A_{23}	16.1087	0.217189	16.8805	104.8	4.8	f	106.4
			$\bar{x}$ = 16.8199 ± 0.0809 (s)				
			0.5% RSD				
B_{31}	34.9872	0.467080	36.5188	104.4	4.4	0.453466	103.0
B_{32}	34.9872	0.468553	36.6345	104.7	4.7	f	103.3
[g]B_{33}	34.9872	0.420772	NA	NA	NA	f	NA
			$\bar{x}$ = 36.5767 ± 0.0579 (d)				
			0.2% RSD				
A_{41}	85.9131	1.093007	85.7088	99.8	–0.2	1.151505	94.9
A_{42}	85.9131	1.117038	87.5973	102.0	2.0	f	97.0
A_{43}	85.9131	1.033373	81.0223	94.3	–5.7	f	89.7
			$\bar{x}$ = 84.7761 ± 3.3853 (s)				
			4.0% RSD				
B_{51}	174.936	2.276200	178.693	102.1	2.1	2.427995	93.7
B_{52}	174.936	2.222198	174.449	99.7	–0.3	f	91.5
B_{53}	174.936	2.206976	173.253	99.0	–1.0	f	90.9
			$\bar{x}$ = 175.465 ± 2.859 (s)				
			1.6% RSD				
A_{61}	268.478	3.350311	263.105	98.0	–2.0	3.758757	89.1
A_{62}	268.478	3.426546	269.096	100.2	0.2	f	91.2
A_{63}	268.478	3.438818	270.060	100.6	0.6	f	91.5
			$\bar{x}$ = 267.420 ± 3.768 (s)	$\bar{x}$= 100.0 ± 4.0 (s)			$\bar{x}$ = 99.7 ± 9.1 (s)
			1.4% RSD	4.0% RSD (n = 17)			9.1% RSD (n = 17)
LOD	0.05958 mg/mL						
LOQ	0.19860 mg/mL						
ELOQ	1.64389 mg/mL						

[a] Peak Area Ratio (PAR) = EHMC peak area/IS peak area.
[b] Determined Conc. (mg/mL) = ((PAR – y-intercept)/slope) * (density/1 g) * (0.025 dilution factor).
[c] % D/E = (D/E) * (100).
[d] % Relative Error = [(D–E)/E] * (100).
[e] % Recovery = ($PAR_{Spiked\ matrix\ std}$ /$PAR_{solvent\ std}$) * (100).
[f] A single solvent standard was prepared at each concentration.
[g] This sample was rejected by the Q-test at the 90% confidence level and is not included in the calculations.
NA = not applicable.

Table 11. Summary of System Suitability Results

Day of analysis	EHMC theoretical plates (N)[a]	EHMC peak tailing (T)[a]	IS theoretical plates (N)[b]	IS peak tailing (T)[b]	Resolution	Precision (% RSD)
GC method criteria	≥ 200,000	0.6 ≤ T ≤ 1.2	≥ 400,000	0.6 ≤ T ≤ 1.2	≥ 40	≤ 2.0
Method validation	298,295.75	0.984	634,948.03	0.988	49.507	1.2
Stability study results						
Day 0	301,789.07	0.990	625,770.87	1.002	49.460	0.4
Day 7	299,806.86	0.987	595,247.53	0.986	48.446	0.5
Day 21	320,078.22	0.983	562,849.57	1.003	48.687	0.6
Day 35	332,594.19	1.003	498,417.84	0.991	48.120	0.9
Day 42	327,196.76	0.989	500,031.28	0.998	47.842	1.3

[a] EHMC = 2-ethylhexyl p-methoxycinnamate.
[b] IS = Benzyl benzoate.

Table 12. Summary of Spiked Matrix Standard Curve Parameters Determined During the 42-Day Stability Study

	Day 0	Day 7	Day 21	Day 35	Day 42
Correlation coefficient	0.999999	0.999970	0.999986	0.999955	0.999807
Slope	0.000291838	0.000315540	0.000297656	0.000310157	0.000299540
Y-intercept	0.000616486	–0.000800073	–0.00320471	0.00148697	–0.00254658

Table 13. Results of Homogeneity Evaluation of EHMC in Corn Oil (~ 200 mg/mL)

Sample	Expected (E) concentration (mg/mL)	Determined (D) concentration (mg/mL)	% D/E	% D/Z[b]
HT_1	200.238	195.297	97.5	97.9
HT_2	200.238	196.544	98.2	98.5
HT_3	200.238	196.465	98.1	98.5
		$\bar{x}$ = 196.102 ± 0.698 (s)	$\bar{x}$ = 97.9 ± 0.4 (s)	$\bar{x}$ = 98.3 ± 0.3 (s)
		% RSD = 0.4	% RSD = 0.4	% RSD = 0.3
HM_1	200.238	198.880	99.3	99.7
HM_2	200.238	201.963	100.9	101.2
HM_3	200.238	202.748	101.3	101.6
		$\bar{x}$ = 201.197 ± 2.045 (s)	$\bar{x}$ = 100.5 ± 1.1 (s)	$\bar{x}$= 100.8 ± 1.0 (s)
		% RSD = 1.0	% RSD = 1.1	% RSD = 1.0
HB_1	200.238	201.188	100.5	100.8
HB_2	200.238	199.975	99.9	100.2
HB_3	200.238	202.401	101.1	101.5
		$\bar{x}$ = 201.188 ± 1.213 (s)	$\bar{x}$ = 100.5 ± 0.6 (s)	$\bar{x}$ = 100.8 ± 0.7 (s)
		% RSD = 0.6	% RSD = 0.6	% RSD = 0.7
	Day 0 value[a]	$\bar{x}$ = 199.496 ± 2.831 (s)		
		% RSD = 1.4		

[a] Determined as the mean of the nine determined concentrations.

[b] Day 0 determined value (Z) = 199.496 mg/mL.

T = top.
M = middle.
B = bottom.

Table 14. Results of Homogeneity Evaluation of EHMC in Corn Oil (~ 20 mg/mL)

Sample	Expected (E) concentration (mg/mL)	Determined (D) concentration (mg/mL)	% D/E
LT_1	20.6200	20.3809	98.8
LT_2	20.6200	20.2976	98.4
LT_3	20.6200	20.2718	98.3
		$\bar{x}$ = 20.3168 ± 0.0570 (s)	$\bar{x}$ = 98.5 ± 0.3 (s)
		% RSD = 0.3	% RSD = 0.3
LM_1	20.6200	20.4807	99.3
LM_2	20.6200	20.3241	98.6
LM_3	20.6200	20.1431	97.7
		$\bar{x}$ = 20.3160 ± 0.1689 (s)	$\bar{x}$ = 98.5 ± 0.8 (s)
		% RSD = 0.8	% RSD = 0.8
LB_1	20.6200	20.3127	98.5
LB_2	20.6200	20.2103	98.0
LB_3	20.6200	20.4834	99.3
		$\bar{x}$ = 20.3355 ± 0.1380 (s)	$\bar{x}$ = 98.6 ± 0.7 (s)
		% RSD = 0.7	% RSD = 0.7
		$\bar{x}$ = 20.3227 ± 0.1131 (s)	
		% RSD = 0.6	

T = top.
M = middle.
B = bottom.

Table 15. Results for the 3-Hour Simulated Dosing Study of EHMC in Corn Oil (~ 20 mg/mL)

Sample	Expected (E) concentration (mg/mL)	Determined (D) concentration (mg/mL)	% D/E	% D/Z[b]
$3DT_1$	19.9542	19.4389	97.4	100.7
$3DT_2$	19.9542	19.3797	97.1	100.4
$3DT_3$	19.9542	19.3404	96.9	100.2
		$\bar{x}$ = 19.3863 ± 0.0496 (s) % RSD = 0.3	$\bar{x}$ = 97.1 ± 0.3 (s) % RSD = 0.3	$\bar{x}$ = 100.4 ± 0.3 (s) % RSD = 0.3
$3DM_1$	19.9542	19.2990	96.7	100.0
$3DM_2$	19.9542	19.5180	97.8	101.1
$3DM_3$	19.9542	19.3547	97.0	100.3
		$\bar{x}$ = 19.3906 ± 0.1138 (s) % RSD = 0.6	$\bar{x}$ = 97.2 ± 0.6 (s) % RSD = 0.6	$\bar{x}$ = 100.5 ± 0.6 (s) % RSD = 0.6
$3DB_1$	19.9542	19.0400	95.4	98.6
$3DB_2$	19.9542	19.3537	97.0	100.3
$3DB_3$	19.9542	18.9848	95.1	98.4
		$\bar{x}$ = 19.1262 ± 0.1990 (s) % RSD = 1.0	$\bar{x}$ = 95.8 ± 1.0 (s) % RSD = 1.0	$\bar{x}$ = 99.1 ± 1.0 (s) % RSD = 1.0
	Day 0 value[a]	$\bar{x}$ = 19.3010 ± 0.1759 (s) % RSD = 0.9		

[a] Determined as the mean of the nine determined concentrations.
[b] Day 0 determined value (Z) = 19.3010 mg/mL.
T = top.
M = middle.
B = bottom.

Table 16. Stability Results of EHMC in Corn Oil (~ 20 mg/mL) Under Ambient Conditions

Time point	Sample	Expected (E) concentration (mg/mL)	Determined (D) concentration (mg/mL)	% D/E	% D/Z[a]
Day 7	L7-A1	19.9542	19.3788	97.1	100.4
	L7-A2	19.9542	19.4687	97.6	100.9
	L7-A3	19.9542	19.3419	96.9	100.2
			$\bar{x}$ = 19.3965 ± 0.0652 (s)	$\bar{x}$ = 97.2 ± 0.4 (s)	$\bar{x}$ = 100.5 ± 0.4 (s)
			% RSD = 0.3	% RSD = 0.4	% RSD = 0.4
Day 21	L21-A1	19.9542	20.1093	100.8	104.2
	L21-A2	19.9542	19.7696	99.1	102.4
	L21-A3	19.9542	20.1286	100.9	104.3
			$\bar{x}$ = 20.0025 ± 0.2019 (s)	$\bar{x}$ = 100.3 ± 1.0 (s)	$\bar{x}$ = 103.6 ± 1.1 (s)
			% RSD = 1.0	% RSD = 1.0	% RSD = 1.1
Day 35	L35-A1	19.9542	19.3453	96.9	100.2
	L35-A2	19.9542	19.4473	97.5	100.8
	L35-A3	19.9542	19.3885	97.2	100.5
			$\bar{x}$ = 19.3937 ± 0.0512 (s)	$\bar{x}$ = 97.2 ± 0.3 (s)	$\bar{x}$ = 100.5 ± 0.3 (s)
			% RSD = 0.3	% RSD = 0.3	% RSD = 0.3
Day 42	L42-A1	19.9542	19.3212	96.8	100.1
	L42-A2	19.9542	18.8916	94.7	97.9
	L42-A3	19.9542	19.0605	95.5	98.8
			$\bar{x}$ = 19.0911 ± 0.2164 (s)	$\bar{x}$ = 95.7 ± 1.1 (s)	$\bar{x}$ = 98.9 ± 1.1 (s)
			% RSD = 1.1	% RSD = 1.1	% RSD = 1.1

[a] Day 0 determined value (Z) = 19.3010 mg/mL.

Table 17. Stability Results of EHMC in Corn Oil (~ 20 mg/mL) Under Refrigerated Conditions

Time point	Sample	Expected (E) concentration (mg/mL)	Determined (D) concentration (mg/mL)	% D/E	% D/Z[a]
Day 7	L7-R1	19.9542	19.1926	96.2	99.4
	L7-R2	19.9542	19.3404	96.9	100.2
	L7-R3	19.9542	19.4301	97.4	100.7
			$\bar{x}$ = 19.3210 ± 0.1199 (s)	$\bar{x}$ = 96.8 ± 0.6 (s)	$\bar{x}$ = 100.1 ± 0.7 (s)
			% RSD = 0.6	% RSD = 0.6	% RSD = 0.7
Day 21	L21-R1	19.9542	20.0197	100.3	103.7
	L21-R2	19.9542	19.9564	100.0	103.4
	L21-R3	19.9542	20.0558	100.5	103.9
			$\bar{x}$ = 20.0106 ± 0.0503 (s)	$\bar{x}$ = 100.3 ± 0.3 (s)	$\bar{x}$ = 103.7 ± 0.3 (s)
			% RSD = 0.3	% RSD = 0.3	% RSD = 0.3
Day 35	L35-R1	19.9542	19.4596	97.5	100.8
	L35-R2	19.9542	19.6244	98.3	101.7
	L35-R3	19.9542	20.8088	104.3	107.8
			$\bar{x}$ = 19.9643 ± 0.7360 (s)	$\bar{x}$ = 100.0 ± 3.7 (s)	$\bar{x}$ = 103.4 ± 3.8 (s)
			% RSD = 3.7	% RSD = 3.7	% RSD = 3.7
Day 42	L42-R1	19.9542	19.1487	96.0	99.2
	L42-R2	19.9542	19.5227	97.8	101.1
	L42-R3	19.9542	19.0265	95.4	98.6
			$\bar{x}$ = 19.2326 ± 0.2585 (s)	$\bar{x}$ = 96.4 ± 1.2 (s)	$\bar{x}$ = 99.6 ± 1.3 (s)
			% RSD = 1.3	% RSD = 1.2	% RSD = 1.3

[a] Day 0 determined value (Z) = 19.3010 mg/mL.

Table 18. Stability Results of EHMC in Corn Oil (~ 200 mg/mL) Under Ambient Conditions

Time point	Sample	Expected (E) Concentration (mg/mL)	Determined (D) Concentration (mg/mL)	% D/E	% D/Z[a]
Day 7	H7-A1	200.238	199.872	99.8	100.2
	H7-A2	200.238	199.194	99.5	99.8
	H7-A3	200.238	200.112	99.9	100.3
			$\bar{x}$ = 199.726 ± 0.476 (s)	$\bar{x}$ = 99.7 ± 0.2 (s)	$\bar{x}$ = 100.1 ± 0.3 (s)
			% RSD = 0.2	% RSD = 0.2	% RSD = 0.3
Day 21	H21-A1	200.238	203.000	101.4	101.8
	H21-A2	200.238	202.023	100.9	101.3
	H21-A3	200.238	199.525	99.6	100.0
			$\bar{x}$ = 201.516 ± 1.792 (s)	$\bar{x}$ = 100.6 ± 0.9 (s)	$\bar{x}$ = 101.0 ± 0.9 (s)
			% RSD = 0.9	% RSD = 0.9	% RSD = 0.9
Day 35	H35-A1	200.238	197.715	98.7	99.1
	H35-A2	200.238	197.464	98.6	99.0
	H35-A3	200.238	196.045	97.9	98.3
			$\bar{x}$ = 197.075 ± 0.901 (s)	$\bar{x}$ = 98.4 ± 0.4 (s)	$\bar{x}$ = 98.8 ± 0.4 (s)
			% RSD = 0.5	% RSD = 0.4	% RSD = 0.4
Day 42	H42-A1	200.238	196.427	98.1	98.5
	H42-A2	200.238	198.697	99.2	99.6
	H42-A3	200.238	202.340	101.0	101.4
			$\bar{x}$ = 199.155 ± 2.983 (s)	$\bar{x}$ = 99.4 ± 1.5 (s)	$\bar{x}$ = 99.8 ± 1.5 (s)
			% RSD = 1.5	% RSD = 1.5	% RSD = 1.5

[a] Day 0 determined value (Z) = 199.496 mg/mL.

Table 19. Stability Results of EHMC in Corn Oil (~ 200 mg/mL) Under Refrigerated Conditions

Time point	Sample	Expected (E) concentration (mg/mL)	Determined (D) concentration (mg/mL)	% D/E	% D/Z[a]
Day 7	H7-R1	200.238	199.295	99.5	99.9
	H7-R2	200.238	199.800	99.8	100.2
	H7-R3	200.238	199.626	99.7	100.1
			$\bar{x}$ = 199.574 ± 0.257 (s)	$\bar{x}$ = 99.7 ± 0.2(s)	$\bar{x}$ = 100.1 ± 0.2 (s)
			% RSD = 0.1	% RSD = 0.2	% RSD = 0.2
Day 21	H21-R1	200.238	205.201	102.5	102.9
	H21-R2	200.238	203.521	101.6	102.0
	H21-R3	200.238	203.293	101.5	101.9
			$\bar{x}$ = 204.005 ± 1.042 (s)	$\bar{x}$ = 101.9 ± 0.6 (s)	$\bar{x}$ = 102.3 ± 0.6 (s)
			% RSD = 0.5	% RSD = 0.6	% RSD = 0.6
Day 35	H35-R1	200.238	195.722	97.7	98.1
	H35-R2	200.238	196.000	97.9	98.2
	H35-R3	200.238	195.976	97.9	98.2
			$\bar{x}$ = 195.899 ± 0.154 (s)	$\bar{x}$ = 97.8 ± 0.1 (s)	$\bar{x}$ = 98.2 ± 0.1 (s)
			% RSD = 0.1	% RSD = 0.1	% RSD = 0.1
Day 42	H42-R1	200.238	195.710	97.7	98.1
	H42-R2	200.238	195.460	97.6	98.0
	H42-R3	200.238	197.291	98.5	98.9
			$\bar{x}$ = 196.154 ± 0.993 (s)	$\bar{x}$ = 97.9 ± 0.5 (s)	$\bar{x}$ = 98.3 ± 0.5 (s)
			% RSD = 0.5	% RSD = 0.5	% RSD = 0.5

[a] Day 0 determined value (Z) = 199.496 mg/mL.

Table 20. Results of High Dose Method Verification Samples of EHMC in Corn Oil (~ 400 mg/mL)

Sample	Expected (E) concentration (mg/mL)	Determined (D) concentration (mg/mL)	% D/E
HD1	404.847	406.454	100.4
HD2	404.847	404.797	100.0
HD3	404.847	403.334	99.6
		$\bar{x}$ = 404.862 ± 1.561 (s)	$\bar{x}$ = 100.0 ± 0.4 (s)
		% RSD = 0.4	% RSD = 0.4

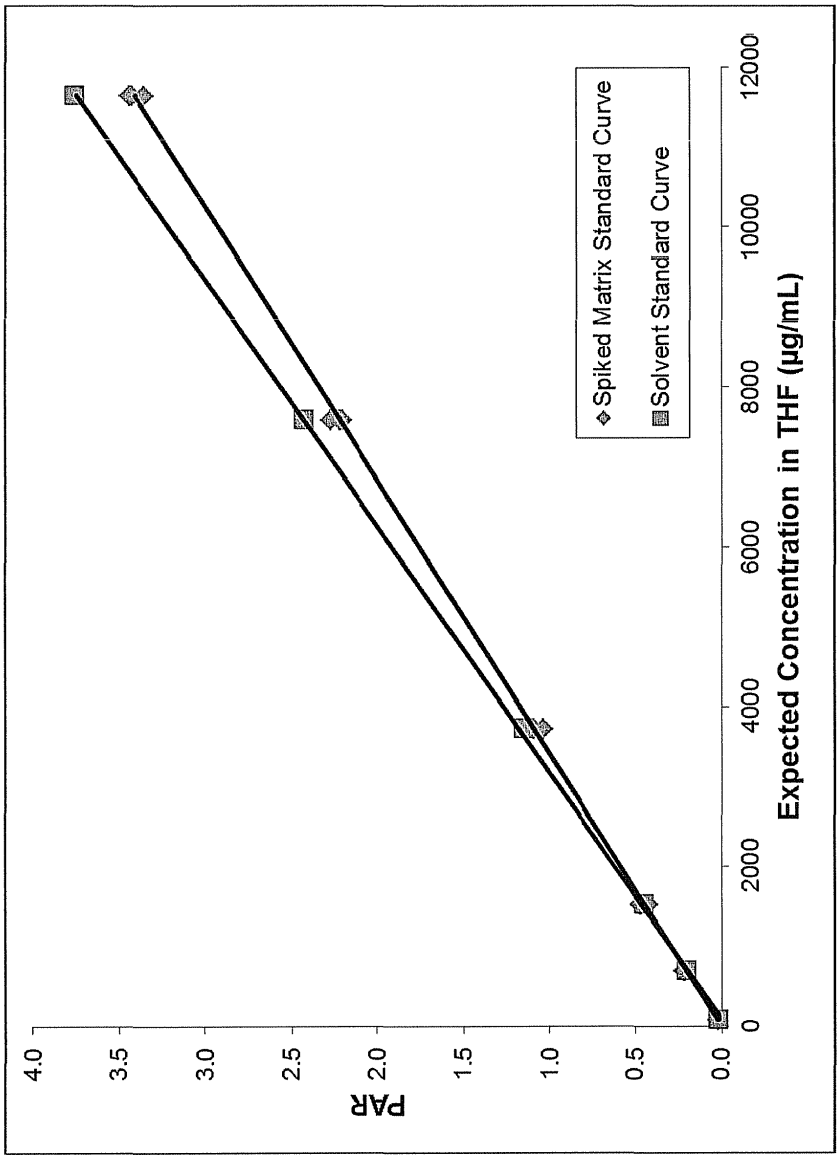


Figure 1. Solvent Curve and Spiked Matrix Standard Curve for the Method Validation of EHMC in Corn Oil

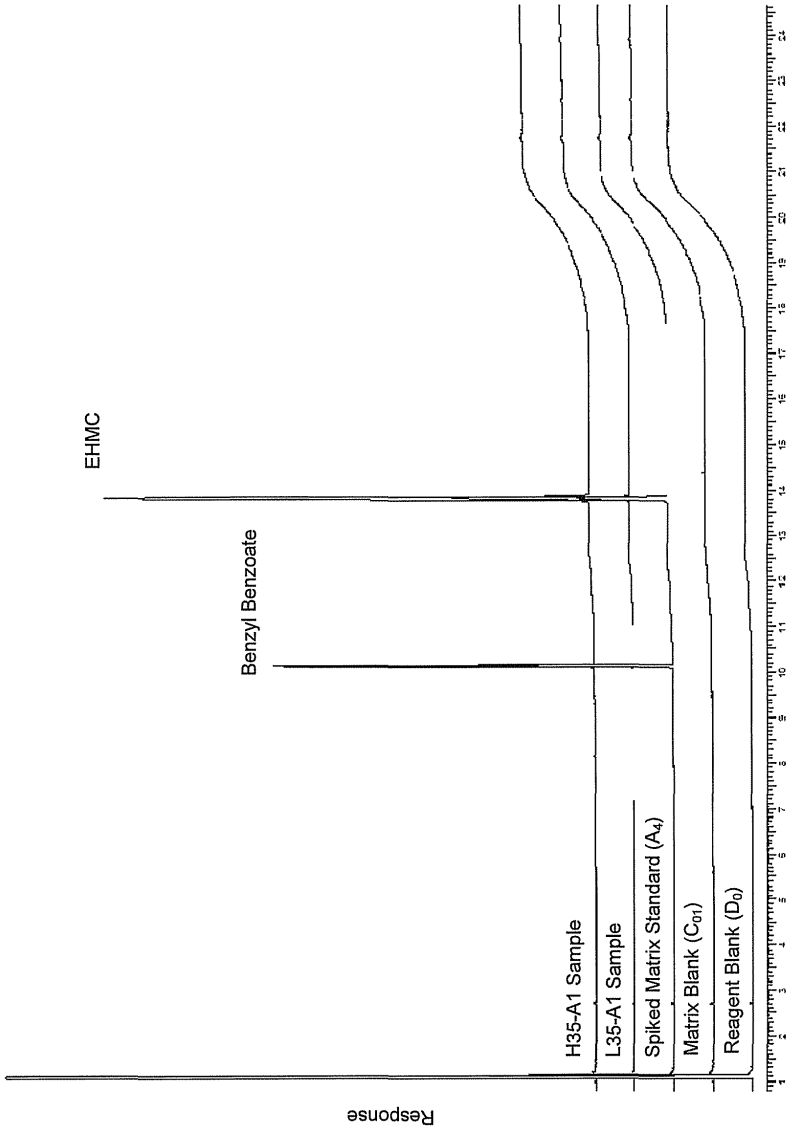


Figure 2. Representative GC Chromatograms for EHMC in Corn Oil, H35-A1 Sample (~ 200 mg/mL), L35-A1 Sample (~ 20 mg/mL), Spiked Matrix Standard A_4 (~ 86 mg/mL), Matrix Blank (C_{01}), and Reagent Blank (D_0)

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30

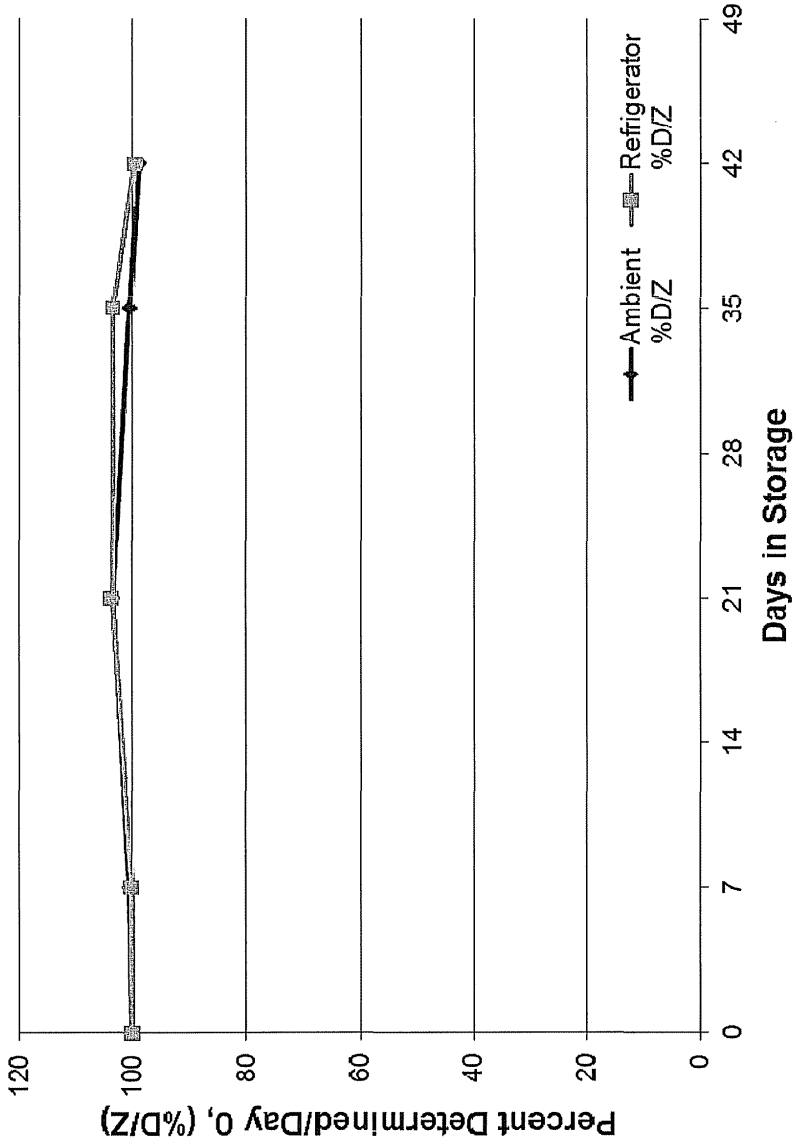

Figure 3. Summary of 42-Day Stability Study of EHMC in Corn Oil (~ 20 mg/mL)

31

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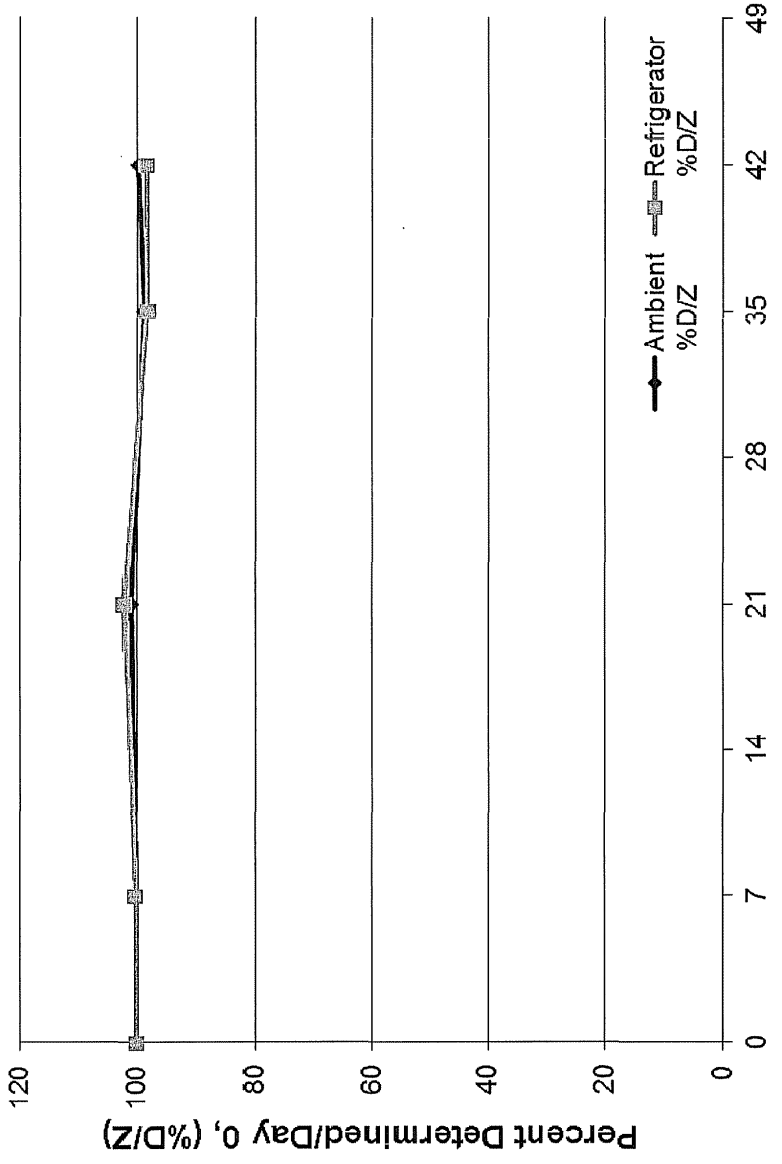

Figure 4. Summary of 42-Day Stability Study of EHMC in Corn Oil (~ 200 mg/mL)

32

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Dose Formulation Development Study of 2-Ethylhexyl p-methoxycinnamate in Corn Oil

Appendix A: Data Summary

NIEHS Contract No. HHSN273201100001C
NTP ChemTask No. CHEM10992
MRI Project No. 110730
MRI Assignment No. 2010

Appendix A: Data Summary

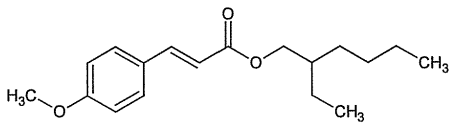
1. Introduction

In anticipation of a gavage study, the purpose of this dose formulation study was to develop and validate a method for the quantitation of 2-ethylhexyl p-methoxycinnamate (EHMC) in corn oil. The method validation was conducted for the quantitation of EHMC in corn oil encompassing a formulation range of ~ 1.7 to ~ 268 mg/mL with a high dose method verification performed at ~ 400 mg/mL. The study also included homogeneity evaluations and 42-day forward storage stability studies of two EHMC formulations in corn oil at ~ 20 mg/mL and ~ 200 mg/mL. A 3-hour simulated dosing study was performed on an ~ 20 mg/mL formulation. This study was initiated on December 9, 2010.

2. Chemical Information

Test Article:	2-Ethylhexyl p-methoxycinnamate (EHMC), stabilized with 0.09% butylated hydroxytoluene (BHT)
Lot No.:	A0293319
MRI-Assigned Batch No.:	01
Supplier:	Acros Organics
Purity:	99.8% (per C of A)
Molecular Formula:	$C_{18}H_{26}O_3$
Molecular Weight:	290.39
CAS No.:	5466-77-3

Structure:



3. Materials and Equipment

GC system equipped with an Agilent 6890N gas chromatograph with Agilent 7683 autosampler, with FID detector, and TotalChrom data system, Version 6.3.0
Column, DB-5 (Agilent), 30 m × 0.53 mm ID, 1.5-µm film thickness
Benzyl benzoate, 99.0% purity, Sigma, used as internal standard
Tetrahydrofuran (THF), Honeywell, High Purity grade
Corn oil, Spectrum Chemical
Low actinic volumetric glassware, Class A, as needed
Amber GC vials with crimp caps, National Scientific

Amber serum vial, ~ 50 mL, with crimp caps, Kimble Chase
Balances: Mettler Toledo, Model: XS205DU
Mettler Toledo, Model: AG285
Mettler Toledo, Model: XS204

4. Method Validation

Method validation was performed using a GC/FID method for the analysis of EHMC in corn oil that covered a formulation range of ~ 1.7 to ~ 268 mg/mL (or an analytical concentration range of ~ 76 to ~ 11,600 µg/mL in THF). The method validation evaluated the linearity, precision, and accuracy of the prepared spiked matrix standards. Percent recovery of EHMC was determined by comparing the response of the spiked matrix standards to the solvent standards at equivalent concentrations. Spiked matrix and solvent standard curves were prepared at six concentrations to cover the analytical range; triplicate preparations at each concentration level were used for the spiked matrix standard curve.

The solvent standards, spiked matrix standards, and blanks were analyzed using the GC system outlined in Table A-1.

The spiked matrix standard curve proved to be linear ($r \geq 0.999$), precise (% RSD ≤ 4.0), and accurate (% RE from – 7.2% to 4.8%). The mean %D/E for the spiked matrix standards (n = 17; spiked matrix standard B_{33} was rejected by the Q-test at the 90% confidence level) was 100.0% ± 4.0 (s). The overall percent recovery for the spiked matrix standards relative to solvent standards at the same concentration was 99.7% ± 9.1 (s) (n = 17; spiked matrix standard B_{33} was rejected by the Q-test at the 90% confidence level). The estimated LOD for the method was 0.05958 mg/mL, the estimate LOQ was 0.19860 mg/mL, and the ELOQ was 1.64389 mg/mL [% RSD = 1.2% (n = 3)]. The data from the method validation are presented graphically in Figure A-1.

The analytical system described in Table A-1 was evaluated for system precision, theoretical plates, peak tailing, and resolution according to USP guidelines.[1] System precision was calculated using the mean PAR determined from six replicate injections of a mid-range spiked matrix standard. Peak tailing (at 5% peak height), theoretical plates (tangential method), and resolution were evaluated from an injection of a mid-range spiked matrix standard. The system suitability results for EHMC are shown in Table A-2.

[1] *United States Pharmacopeia* [621] Chromatography, (2008), official from May 1, 2008, 31st Edition, Volume 1, pp. 232-243.

Table A-1. GC Conditions for Method Validation

Gas Chromatograph:	Agilent 6890N gas chromatograph with Agilent 7683 autosampler
Column:	DB-5 (Agilent), 30 m × 0.53 mm ID,1.5-µm film thickness
Liner Type:	Dual Tapered with glass wool
Injector Temperature:	280°C
Injector Mode:	Split, Split Ratio 75:1
Detector:	Flame Ionization Detector (FID)
Detector Temperature:	300°C
Detector Range:	1
Carrier Gas:	Helium
Carrier Gas Flow Rate:	~ 10.0 mL/min
Hydrogen Flow:	~ 30 mL/min
Air Flow:	~ 300 mL/min
Make-up Gas:	Nitrogen
Make-up Gas Flow Rate:	~ 25 mL/min
Injection Volume:	1 µL
Oven Program:	85°C (1-min hold), 15.0°C/min to 255°C (5-min hold), 15°C/min to 300°C (5-min hold)
Run Time:	25 min
Data System:	TotalChrom, Version 6.3.0
Retention Times:	EHMC: ~ 14.6 min Benzyl benzoate (IS): ~ 10.6 min

Table A-2. System Suitability Results for Method Validation

System precision	Theoretical plates	Tailing factor	Resolution
Analytical results: % RSD = 1.2 (n = 6)	EHMC = 298,295.75 IS = 634,948.03	EHMC = 0.984 IS = 0.988	49.507
Method criteria: % RSD ≤ 2.0	EHMC ≥ 200,000 IS ≥ 400,000	0.6 ≤ T ≤ 1.2 0.6 ≤ T ≤ 1.2	≥ 40

5. High Dose Method Verification at ~ 400 mg/mL EHMC in Corn Oil

A high dose formulation of EHMC in corn oil was prepared at 404.847 mg/mL. This formulation was diluted with corn oil in order to show that a high dose formulation could be diluted into the validated curve range with a ≤ 0.4 % RSD.

6. 42-Day Stability Study of EHMC in Corn Oil (Forward Method)

Formulations of EHMC were prepared at concentrations of ~ 20 and ~ 200 mg/mL in corn oil. The formulations were stored under ambient and refrigerated conditions for a 42-day forward stability study. The formulations were removed from storage and prepared for analysis on Days 7, 21, 35, and 42.

When compared to the Day 0 determined low dose concentration of 19.3010 mg/mL, the results of the 42-day stability study for the ~ 20-mg/mL formulation indicated losses of ≤ 1.1% under ambient conditions and ≤ 0.4% under refrigerated conditions. Statistical analysis used to determine the test variability limit, established that in order for the loss of EHMC to be statistically significant at the 95% confidence level, the loss must be greater than 1.9% at ambient conditions when compared to Day 0 and greater than 2.7% at refrigerated conditions when compared to Day 0. It was concluded that the low dose formulation can be stored for 42 days under ambient conditions with mean recoveries ≥ 98.9% or 42 days under refrigerated conditions with recoveries ≥ 99.6%. The storage stability results are presented in Figure A-2.

When compared to the Day 0 determined high dose concentration of 199.496 mg/mL, the results of the 42-day stability study for the ~ 200-mg/mL formulation indicated losses of ≤ 1.2% under ambient conditions and ≤ 1.8% under refrigerated conditions. Statistical analysis used to determine the test variability limit, established that in order for the loss of EHMC to be statistically significant at the 95% confidence level, the loss must be greater than 2.7% at ambient conditions when compared to Day 0 and greater than 2.5% at refrigerated conditions when compared to Day 0. It was concluded that the high dose formulation can be stored for 42 days under ambient conditions with mean recoveries ≥ 98.8% or 42 days under refrigerated conditions with recoveries ≥ 98.2%. The storage stability results are presented in Figure A-3.

7. 3-Hour Simulated Dosing Study of ~ 20 mg/mL EHMC in Corn Oil

On Day 0, a portion (~ 50 mL) of the prepared ~ 20-mg/mL dose formulation was transferred into a clear 50-mL serum vial, exposed to light and air, and stirred. To simulate dosing conditions, a 1-mL aliquot of the formulation was removed at 15-minute intervals and discarded. After 3 hours, triplicate ~ 1-g portions from each of the top, middle, and bottom locations of the formulation were accurately weighed into individual, 25-mL volumetric flasks. A 2-mL aliquot of IS solution was added to each flask. The contents of the flasks were diluted to volume with THF and mixed well. The results of the 3-hour simulated dosing study showed mean % D/E values ≥ 95.8%.

8. Homogeneity Evaluation of Low Dose ~ 20 mg/mL EHMC in Corn Oil

A formulation of ~ 20 mg/mL EHMC in corn oil was evaluated for homogeneity and the results indicated the formulation was homogenous (0.6% RSD).

9. Homogeneity Evaluation of High Dose ~ 200 mg/mL EHMC in Corn Oil

A formulation of ~ 200 mg/mL EHMC in corn oil was evaluated for homogeneity and the results indicated the formulation was homogenous (1.4% RSD).

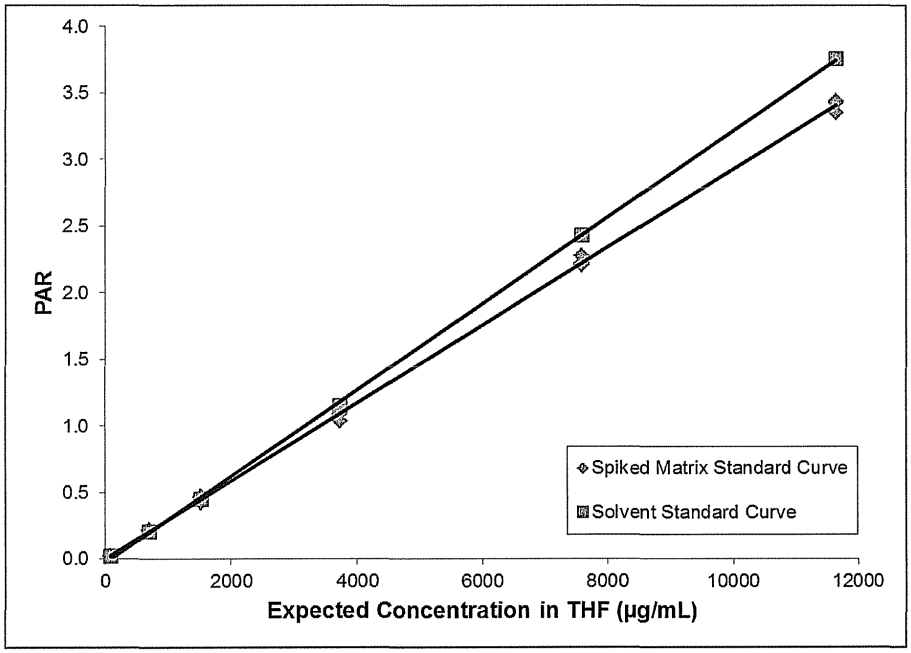


Figure A-1. Solvent Curve and Spiked Matrix Standard Curve for the Method Validation of EHMC in Corn Oil

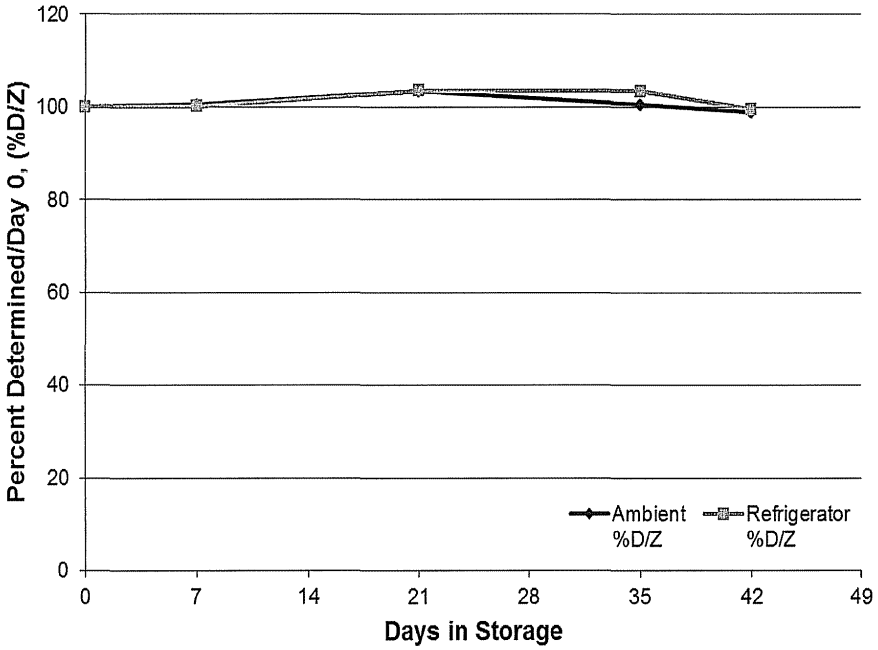


Figure A-2. Summary of 42-Day Stability Study of EHMC in Corn Oil (~ 20 mg/mL)

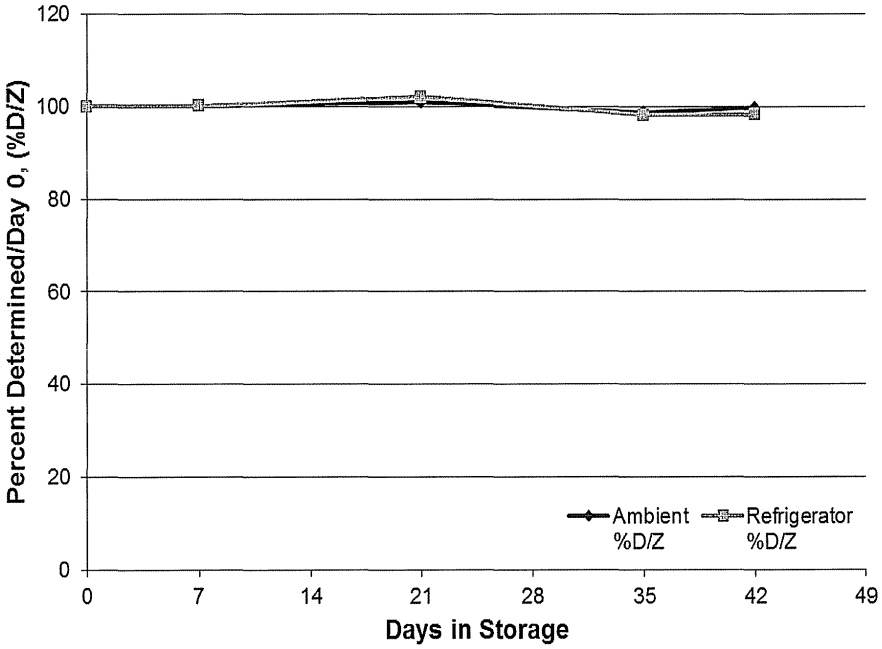


Figure A-3. Summary of 42-Day Stability Study of EHMC in Corn Oil (~ 200 mg/mL)

Dose Formulation Development Study of 2-Ethylhexyl p-methoxycinnamate in Corn Oil

Appendix B: Formulation Preparation and Analysis Procedures for Toxicology Laboratories

NIEHS Contract No. HHSN273201100001C
NTP ChemTask No. CHEM10992
MRI Project No. 110730
MRI Assignment No. 2010

Appendix B: Formulation Preparation and Analysis Procedures for Toxicology Laboratories

1. Introduction

This appendix contains procedures for the method validation, formulation preparation, formulation storage, and formulation analysis of EHMC in corn oil from ~ 1.7 to ~ 268 mg/mL.

2. Materials and Equipment

The materials and equipment listed below, or their equivalent, are to be used for the preparation and assay of the formulations.

GC system equipped with an Agilent 6890N gas chromatograph with Agilent 7683 autosampler, with FID detector, and TotalChrom data system, Version 6.3.0
Column, DB-5 (Agilent), 30 m × 0.53 mm ID, 1.5-µm film thickness
Benzyl benzoate, 99.0% purity, Sigma, used as internal standard
Tetrahydrofuran (THF), Honeywell, High Purity grade
Corn oil, Spectrum Chemical
Low actinic volumetric glassware, Class A, as needed
Amber GC vials with crimp caps, National Scientific
Amber serum vial, ~ 50 mL, with crimp caps, Kimble Chase
Balances: Mettler Toledo, Model: XS205DU
Mettler Toledo, Model: AG285
Mettler Toledo, Model: XS204

3. Method Validation

3.1 Preparation of Standards

3.1.1 Internal Standard Solution

Prepare an internal standard (IS) solution by accurately weighing and transferring ~ 4,000 mg of benzyl benzoate into a 100-mL volumetric flask, diluting the contents of the flask to volume with THF, and mixing well. Expected IS concentration: ~ 40 mg/mL.

3.1.2 Stock Solutions

Prepare two stock solutions of EHMC by accurately weighing and transferring ~ 2,900 mg (Stock A) and ~ 1,900 mg (Stock B) into two individual 50-mL volumetric flasks. Dilute the contents of each flask to volume with THF and mix well. Expected concentrations: Stock A ~ 58 mg/mL; and Stock B ~ 38 mg/mL.

3.1.3 Intermediate Standard Solutions

Following the dilution scheme in Table B-1, prepare six intermediate standard solutions (IB_1 to IA_6) of EHMC in THF by transferring aliquots from alternating Stock Solutions A and B, diluting the contents of the flask to volume with THF, and mix well.

Table B-1. Preparation of Intermediate Standard Solutions for Method Validation

Intermediate standard solution	Stock solution	Stock solution aliquot (mL)	Final volume (mL)	Expected analytical concentration (~ mg/mL)
IB_1	B	1	100	0.38
IA_2	A	3	50	3.5
IB_3	B	5	25	7.6
IA_4	A	8	25	19
[a] IB_5	B	NA	NA	38
[b] IA_6	A	NA	NA	58

[a] Use Stock B as IB_5.
[b] Use Stock A as IA_6.

3.1.4 Solvent Standards

Following the dilution scheme in Table B-2, prepare six solvent standards (SB_1 to SA_6 by transferring 5 mL of each intermediate standard solution (IB_1 to IA_6) into individual, 25-mL volumetric flasks containing 2 mL of IS solution. Dilute the contents of each flask to volume with THF and mix well.

Table B-2. Preparation of Solvent Standards for Method Validation

Solvent standard	Intermediate standard solution	Intermediate standard solution aliquot (mL)	IS solution (mL)	Final volume (mL)	Expected analytical concentration (~ µg/mL)
SB_1	IB_1	5	2	25	76
SA_2	IA_2	5	2	25	696
SB_3	IB_3	5	2	25	1,520
SA_4	IA_4	5	2	25	3,712
SB_5	IB_5	5	2	25	7,600
SA_6	IA_6	5	2	25	11,600

3.1.5 Spiked Matrix Standards

Following the dilution scheme in Table B-3, prepare spiked matrix standards (triplicate standards at each concentration) by accurately weighing and transferring ~ 1-g portions of corn oil (~ 0.92 g/mL density) into 18 individual, 25-mL volumetric flasks. Transfer a 5-mL aliquot of each previously prepared intermediate standard solution (IB_1 to IA_6) into the corresponding volumetric flask containing 2 mL of IS solution. Dilute the contents of the flasks to volume with THF and mix well.

Table B-3. Preparation of Spiked Matrix Standards for Method Validation

Spiked matrix standard	Intermediate standard solution	Intermediate standard solution (mL)	Corn oil (~ g)	Final volume (mL)[a]	Expected analytical concentration (~ µg/mL)	Expected formulation concentration (~ mg/mL)
B_{11}, B_{12}, B_{13}	IB_1	5	1	25	76	1.75
A_{21}, A_{22}, A_{23}	IA_2	5	1	25	696	16.0
B_{31}, B_{32}, B_{33}	IB_3	5	1	25	1,520	35.0
A_{41}, A_{42}, A_{43}	IA_4	5	1	25	3,712	85.4
B_{51}, B_{52}, B_{53}	IB_5	5	1	25	7,600	175
A_{61}, A_{62}, A_{63}	IA_6	5	1	25	11,600	267

[a] Contains 2 mL of IS solution.

3.2 Preparation of Blanks

3.2.1 Reagent Blank (D_0)

Use THF as the reagent blank.

3.2.2 IS Blank

Prepare an IS blank by transferring 2 mL of IS solution into a 25-mL volumetric flask, diluting the contents of the flask to volume with THF, and mixing well.

3.2.3 Matrix Blanks

Prepare triplicate matrix blanks without IS (C_{01}, C_{02}, and C_{03}) by accurately weighing and transferring ~ 1-g portions of corn oil (~ 0.92 g/mL density) into individual, 25-mL volumetric flasks, diluting the contents of the flasks to volume with THF, and mixing well.

Prepare a matrix blank with IS (C_{04}) by accurately weighing an ~ 1-g portion of corn oil into a 25-mL volumetric flask, adding 2 mL of IS solution, diluting the contents of the flask to volume with THF, and mixing well.

3.3 Density Determination of Corn Oil

Pre-weigh three, 10-mL volumetric flasks. Fill each flask to volume with corn oil and record the weight. Calculate the density (g/mL) and average density using commonly accepted methods.

3.4 Analysis for Method Validation

Transfer aliquots of each solvent standard, spiked matrix standard, and blank into individual autosampler vials and analyze using the GC system (or equivalent) and parameters described in Table B-4.

Table B-4. GC Conditions for Method Validation

Gas Chromatograph:	Agilent 6890N gas chromatograph with Agilent 7683 autosampler
Column:	DB-5 (Agilent), 30 m × 0.53 mm ID,1.5-µm film thickness
Liner Type:	Dual Tapered with glass wool
Injector Temperature:	280°C
Injector Mode:	Split, Split Ratio 75:1
Detector:	Flame Ionization Detector (FID)
Detector Temperature:	300°C
Detector Range:	1
Carrier Gas:	Helium
Carrier Gas Flow Rate:	~ 10.0 mL/min
Hydrogen Flow:	~ 30 mL/min
Air Flow:	~ 300 mL/min
Make-up Gas:	Nitrogen
Make-up Gas Flow Rate:	~ 25 mL/min
Injection Volume:	1 µL
Oven Program:	85°C (1-min hold), 15.0°C/min to 255°C (5-min hold), 15°C/min to 300°C (5-min hold)
Run Time:	25 min
Data System:	TotalChrom, Version 6.3.0
Retention Times:	EHMC: ~ 14.6 min Benzyl benzoate (IS): ~ 10.6 min

3.5 Calculations of Method Validation

1. Calculate the peak area ratio (PAR) for EHMC as follows:

$$PAR = \frac{\text{Peak Area (EHMC)}}{\text{Peak Area (IS)}}$$

2. Calculate the slope, y-intercept, and correlation coefficient from a weighted (1/x) linear regression analysis of the spiked matrix standard curve by relating the PAR of each spiked matrix standard with its corresponding expected analytical concentration (µg/mL in THF).

3. Use the slope and y-intercept determined for the spiked matrix standard curve and the PAR for each spiked matrix standard to calculate the determined formulation concentration (mg/mL in corn oil) of each spiked matrix standard using the following equation:

$$\text{Determined Concentration (mg/mL)} = \frac{[\text{PAR} - (\text{y-intercept})]}{\text{slope}} \times \frac{\text{d}}{1\text{ g corn oil}} \times \text{DF} \times \frac{1\text{ mg}}{1{,}000\ \mu\text{g}}$$

where: d = density of corn oil (g/mL) = ~ 0.92 g/mL
DF = dilution factor = 25 mL

4. Calculate the slope, y-intercept, and correlation coefficient from a weighted (1/x) linear regression analysis of the solvent standard curve by relating the PAR of each solvent standard with its corresponding expected analytical concentration (µg/mL in THF).
5. Using the slope and y-intercept determined for the solvent standard curve and the PAR for each solvent standard, calculate the determined analytical concentration of each solvent standard using the following equation:

$$\text{Determined concentration (}\mu\text{g/mL)} = \frac{[\text{PAR} - (\text{y-intercept})]}{\text{slope}}$$

6. Evaluate method precision by calculating the percent relative standard deviation (% RSD) of the mean PAR values for the triplicate preparations at each concentration of spiked matrix standard.
7. Calculate method accuracy, expressed as percent relative error (% RE), as follows:

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

where: D = determined formulation concentration (mg/mL)
E = expected formulation concentration (mg/mL)

8. Calculate the percent response recovery for each spiked matrix standard relative to the corresponding solvent standard as follows:

$$\%\text{ Recovery} = \left(\frac{\text{PAR (Spiked matrix stds)}}{\text{PAR (Solvent stds)}}\right) \times 100$$

9. Calculate the limit of detection (LOD) and limit of quantitation (LOQ) based on the standard deviation (s) of the lowest spiked matrix standard expressed as the determined concentration as follows:

$$\text{LOD} = 3 \times \text{s}$$

$$\text{LOQ} = 10 \times \text{s}$$

10. Define the experimental limit of quantitation (ELOQ) as the lowest mean determined concentration (mg/mL in corn oil) of spiked matrix standard with a % RE $\leq \pm$ 10% and a % RSD $\leq$ 10%.

11. Calculate system suitability parameters for system precision, peak tailing (T), theoretical plates (N), and resolution (R) according to USP guidelines.[1] Calculate system precision using the mean PAR determined from six replicate injections of a mid-range spiked matrix standard. Calculate peak tailing (at 5% peak height), theoretical plates (tangential method), and resolution from a single injection of the mid-range spiked matrix standard. System suitability should meet the criteria listed in Table B-5.
12. Calculate sample mean ($\bar{x}$), standard deviation (s), and percent relative standard deviation (% RSD) using commonly accepted techniques.

Table B-5. System Suitability Criteria

System precision[a]	Theoretical plates	Tailing factor	Resolution[b]
Method criteria % RSD ≤ 2.0 (n = 6)	EHMC ≥ 200,000	0.6 ≤ T ≤ 1.2	≥ 40
	IS ≥ 400,000	0.6 ≤ T ≤ 1.2	

[a] Calculate using PAR of mid-range spiked matrix standard.
[b] Between EHMC and IS peaks.

4. Preparation of Formulations

4.1 Preparation of ~ 20 mg/mL Formulation of EHMC in Corn Oil

Prepare a formulation by accurately weighing and transferring ~ 20 g of EHMC into a low actinic, 1-L volumetric flask then adding ~ 200 mL of corn oil. Mix the contents of the flask well. Add additional, ~ 200-mL portions of corn oil and mix well after each addition. Dilute the contents of the flask to volume with corn oil and mix by inversion until a solution is obtained. Finally, mix with a magnetic stir bar for ≥ 5 minutes. Expected EHMC concentration of the formulation: ~ 20 mg/mL.

4.2 Preparation of ~ 200 mg/mL Formulation of EHMC in Corn Oil

Prepare a formulation by accurately weighing and transferring ~ 200 g of EHMC into a low actinic, 1-L volumetric flask then adding ~ 200 mL of corn oil. Mix the contents of the flask well. Add additional, ~ 200-mL portions of corn oil and mix well after each addition. Dilute the contents of the flask to volume with corn oil and mix by inversion until a solution is obtained. Finally, mix with a magnetic stir bar for ≥ 5 minutes. Expected EHMC concentration of the formulation: ~ 200 mg/mL.

[1] *United States Pharmacopeia* [621] Chromatography, (2008), official from May 1, 2008, 31st Edition, Volume 1, pp. 232-243.

4.3 Determination of Formulation Density

For each formulation, pre-weigh three, 10-mL volumetric flasks. Fill each flask to volume with the formulation and record the weight. Calculate the density (g/mL) and average density using commonly accepted methods.

4.4 Storage of Formulations

Formulations of EHMC in corn oil can be stored under ambient (~ 25°C) or refrigerated conditions (~ 5°C) for up to 42 days.

5. Formulation Analysis

This procedure is recommended for the analysis of formulations of EHMC within the concentration range of ~ 1.7 to ~ 268 mg/mL in corn oil. Triplicate portions (~ 1-g each) of formulation are analyzed along with a six-point spiked matrix standard curve. Dose formulations prepared at concentrations exceeding ~ 268 mg/mL may be analyzed, but must be diluted with corn oil to fall within the validated concentration range.

5.1 Preparation of Standards

5.1.1 Internal Standard Solution

Prepare an IS solution as described in Section 3.1.1.

5.1.2 Stock Solutions

Prepare two stock solutions as described in Section 3.1.2.

5.1.3 Intermediate Standard Solutions

Prepare six intermediate standard solutions (IB_1 to IA_6) as described in Section 3.1.3.

5.1.4 Spiked Matrix Standards

Prepare six spiked matrix standards (B_1 to A_6) as described in Section 3.1.5, except prepare each standard with n = 1.

5.2 Blank Preparations

5.2.1 Reagent Blank (D_0)

Use THF as the reagent blank.

5.2.2 IS Blank

Prepare an IS blank as described in Section 3.2.2.

5.2.3 Matrix Blanks

Prepare two matrix blanks, C_{01} and C_{04}, as described in Section 3.2.3.

NOTE: Rename the matrix blank with IS, C_{02}.

5.2.4 Preparation of Formulation Samples for Analysis

Accurately weigh triplicate, ~ 1-g portions of each formulation into individual, 25-mL volumetric flasks containing 2 mL of IS solution. Dilute the contents of each flask to volume with THF and mix well.

Dose formulations that exceed ~ 268 mg/mL should be analyzed after dilution into the validated analytical range. Prepare triplicate samples by accurately weighing ~ 5-g portions of formulation into individual, 50-mL volumetric flasks, diluting the contents of each flask to volume with corn oil, and mixing by inversion. Prepare a second dilution by accurately weighing and transferring an ~ 1-g portion of the first dilution into a 25-mL volumetric flask containing 2 mL of IS solution. Dilute the contents of the flask to volume with THF and mix well. Examples are presented in Table B-6.

Table B-6. Sample Preparation

Formulation concentration (mg/mL)	High dose formulation sample (~ g)	Initial dilution in corn oil (mL)	Formulation aliquot (~ g)	IS solution (mL)	Final dilution (mL)	Expected analytical concentration (~ µg/mL)[a]
Control (0)	NA	NA	1	2	25	0.0
20	NA	NA	1	2	25	800
200	NA	NA	1	2	25	8,000
400	5	50	1	2	25	1,600

[a] The density of each formulation is assumed to be ~ 1.0 g/mL in these calculations only.
NOTE: Determine the density of each formulation and include in the calculations.

5.3 Instrumental System and Parameters

Transfer aliquots of each sample, spiked matrix standard, and blank into individual autosampler vials and analyze using the GC system and parameters described in Section 3.4.

5.4 Calculations

1. Calculate the peak area ratio (PAR) for EHMC as follows:

$$PAR = \frac{\text{Peak Area (EHMC)}}{\text{Peak Area (IS)}}$$

2. Calculate the slope, y-intercept, and correlation coefficient from a weighted (1/x) linear regression analysis of the spiked matrix standard curve by relating the PAR of each spiked matrix standard with its corresponding expected analytical concentration (µg/mL in THF).

3. Using the regression equation from the spiked matrix standard curve and the PAR for each spiked matrix standard, calculate the determined formulation concentration (mg/mL in corn oil) for each spiked matrix standard using the following equation:

$$\text{Determined Concentration (mg/mL)} = \frac{[\text{PAR} - (\text{y-intercept})]}{\text{slope}} \times \frac{\text{d}}{\text{1 g corn oil}} \times \text{DF} \times \frac{\text{1 mg}}{\text{1,000 µg}}$$

where: d = formulation density (g/mL)
DF = dilution factor = 25 mL

4. Calculate the method accuracy, expressed as percent relative error (% RE), as follows:

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

where: D = determined formulation concentration (mg/mL)
E = expected formulation concentration (mg/mL)

5. Using the regression equation form the spiked matrix standard curve and the PAR for each sample, calculate the determined formulation concentration (mg/mL in corn oil) for each sample using the following equation:

$$D = \frac{[\text{PAR (sample)} - (\text{y-intercept})]}{\text{slope}} \times \frac{\text{d}}{\text{Sample Weight (g)}} \times \text{DF} \times \frac{\text{1 mg}}{\text{1,000 µg}}$$

where: D = determined formulation concentration (mg/mL)
d = average formulation density (g/mL)
DF = dilution factor (25 mL)

6. Calculate the mean, standard deviation (s), and percent relative standard deviation (% RSD) of the determined concentrations of each triplicate formulation sample using commonly accepted techniques. If the % RSD > 10%, evaluate the data set for an outlier using the Q-test.

7. Calculate the % of Target for each formulation sample as follows:

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

8. Calculate the mean % of Target for each formulation concentration level as follows:

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

9. Determine if the mean of the formulations is within ± 10% of the designated target concentration. If a formulation is found to be outside the ± 10% tolerance limit, notify the Study Director and/or the Principal Investigator.
10. Calculate system suitability parameters for system precision, peak tailing (T), theoretical plates (N), and resolution (R) according to USP guidelines.[1] Calculate system precision using the mean PAR determined from six replicate injections of a mid-range spiked matrix standard. Calculate peak tailing (at 5% peak height), theoretical plates (tangential method), and resolution from single injections of a mid-range spiked matrix standard. If any parameters do not meet established criteria, contact the Study Director immediately.

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BATTELLE-FA

Analytical Chemistry Services for the NTP
NIH Contract No.: HHSN273201000016C
Battelle Project No.: G006623-EED
NTP ChemTask No.: CHEM11270
CAS No.: 118-60-5

FORMULATION ANALYSIS OF OCTYL SALICYLATE IN CORN OIL

June 3, 2011

Prepared By:

[redacted]
Study Director

Approved By:

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FORMULATION ANALYSIS OF OCTYL SALICYLATE IN CORN OIL

<table>
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11-30-1T 64 mg/mL
11-30-1M 64 mg/mL
11-30-1B 64 mg/mL
11-30-2T 200 mg/mL
11-30-2M 200 mg/mL
11-30-2B 200 mg/mL</td></tr>
<tr><td colspan="2">Battelle Task No.: 16-336-FA-316</td><td>Sample Receipt Date: 5/6/11</td></tr>
<tr><td colspan="2">NTP Task No.: CHEM11270</td><td>Submitter: Integrated Laboratory Systems, Inc. (ILS)</td></tr>
<tr><td colspan="2">Program Supported: TOX</td><td>Study Lab: ILS</td></tr>
<tr><td colspan="2">Analysis Dates: 5/10-5/11/11</td><td>Mix Date: 5/5/11</td></tr>
<tr><td colspan="2">Interim Results Date: 5/12/11</td><td>Receipt Condition: Good</td></tr>
<tr><td colspan="2">SOPs: CSCSPEC.II-050-00, Standard Operating Procedure for the Analysis of Octyl Salicylate Formulations in Corn Oil</td><td>Shipping Container: Seven amber glass vials</td></tr>
<tr><td colspan="2"></td><td>Storage Conditions (@ Battelle): Refrigerated (~5°C)</td></tr>
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 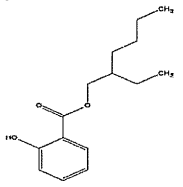 </td><td>Mol. Wt.

250.33 g/mol</td><td>Mol. Formula

$C_{15}H_{22}O_3$</td></tr>
</table>

EXECUTIVE SUMMARY

Formulations of octyl salicylate in corn oil at target concentrations of 0, 64, and 200 mg/mL were prepared by ILS and analyzed by Battelle to determine their concentration and homogeneity prior to administration in support of a TOX study.

The concentrations of all formulations containing octyl salicylate were within 10 percent of target, the National Toxicology Program (NTP) acceptance limit. The relative standard deviation (RSD) values were also within the specified acceptance limit. All formulations were also found to meet all acceptance criteria for homogeneity. The 0 mg/mL formulation contained no detectable octyl salicylate. All other quality criteria stated in the SOP were within acceptance limits.

QUALITY ASSURANCE STATEMENT

FORMULATION ANALYSIS OF OCTYL SALICYLATE IN CORN OIL

NTP ChemTask No.: CHEM11270
Battelle Project No.: G006623-EED
Battelle Task No.: 16-336-FA-316

Listed below are the phases and/or procedures performed by Battelle that were reviewed by the Quality Assurance Unit (QAU) during performance of the task described in this report. Adverse findings, if any, were reported to the study director at the time of review.

Critical Phase Inspected	Date Inspected	Date Reported to Study Director and Management
Formulation analysis	5/10/11	5/10/11
Audit study file	5/25/11	5/25/11
Audit final analytical report	5/25/11	5/25/11

This report reflects the procedures and raw data generated in this study.

In addition to the study-specific audits/inspections cited above, routine inspections of the general facilities and equipment were performed by the QAU and reports were submitted to management as follows:

Facility/Equipment	Date Inspected	Date of Report to Management
Chemistry Technical Center Inspection	12/2, 12/15/08	12/2, 12/22/08
	12/16, 12/23/09	12/16, 12/31/09
	12/28, 12/30/10	12/30/10

[redacted] 6-2-11
Quality Assurance Unit Date

COMPLIANCE STATEMENT

This study was conducted in accordance with the Food and Drug Administration's (FDA's) Good Laboratory Practice (GLP) regulations (21 CFR, Part 58), with the exception of archival of study records at the close of the study. These records are gathered, microfiched, and archived periodically for finalized studies for this program.

[redacted] 6-3-11

Study Director Date

Date Study Initiated (Date Protocol Signed): May 5, 2011
Date Study Completed (Date Final Report Signed): June 3, 2011

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

This report contains:

- A description of the analyses of the formulations for concentration and homogeneity
- Results from the analysis
- Figures
- Conclusions.

This work was performed at Battelle, 505 King Avenue, Columbus, OH 43201, and supports a TOX study.

2.0 FORMULATION SAMPLES

Formulation samples prepared in corn oil (approximately 10 mL each) were received from ILS on May 6, 2011. The samples were formulated on May 5, 2011 with an expiration date of June 17, 2011. They were identified as being from ILS Protocol No. N135-231/232 and had the following concentrations and log numbers.

Table 1. Formulation Samples

Concentration (mg/mL)	ILS Log No.
0	N135-11-94-5511C
64	11-30-1T
64	11-30-1M
64	11-30-1B
200	11-30-2T
200	11-30-2M
200	11-30-2B

All analysis samples that were supplied by ILS in Table 1 were analyzed.

3.0 FORMULATION ANALYSIS FOR CONTENT AND HOMOGENEITY

The formulations were analyzed for octyl salicylate according to SOP CSCSPEC.II-050-00, "Standard Operating Procedure for the Analysis of Octyl Salicylate Formulations in Corn Oil." This SOP was based on work originally conducted under the preliminary chemical studies (PCS) task for octyl salicylate, Battelle Study No. G005430-DYU, NTP ChemTask No. CHEM10883 and the dose formulation developmental (DFD) task for octyl salicylate in corn oil, Battelle Study No. G005430-DZZ, NTP ChemTask No. CHEM10925. The experimental limit of

quantitation (ELOQ) is 0.01 mg/mL, which is the nominal concentration of the lowest standard for this task. This section describes the method, results, and conclusions.

3.1 Method Validation

3.2 Preparation of Diluted Vehicle Solution

The diluted vehicle solution was prepared by adding 1 mL of corn oil to a 100-mL volumetric flask and dissolving it in and diluting the flask to volume with acetone. The flask was sealed and the contents mixed well.

3.3 Preparation of Internal Standard (IS)

IS solution was prepared by weighing approximately 125 mg of benzophenone into a 25-mL volumetric flask and dissolving it in and diluting the flask to volume with acetone. The flask was sealed and the contents mixed well.

3.4 Preparation of Standards and Blanks

3.4.1 Stocks

The amounts of octyl salicylate shown in Table 2 were weighed into individual 50-mL volumetric flasks. The chemical was dissolved in and the flask was diluted to volume with acetone. The flasks were sealed and the contents mixed well.

Table 2. Preparation of Stocks

ID	Target Concentration (mg/mL)	Target Weight (mg)
A	1.25	62.50 ± 2
B	1	50.00 ± 2

3.4.2 Spiking Solutions

The volumes of the A and B indicated in Table 3 were pipetted into individual volumetric flasks. The flasks were diluted to volume with acetone, sealed, and the contents mixed well. A single solution was prepared at all concentrations.

Table 3. Preparation of Spiking Solutions

ID	Target Concentration (mg/mL)	Source	Source Volume (mL)	Final Volume (mL)
C	0.75	A	3	5
D	0.40	B	2	5
E	0.25	A	2	10
F	0.10	B	1	10

3.4.3 Vehicle/Calibration Standards

One (1) mL from each solution A through F was pipetted into individual 10-mL volumetric flasks. One (1) mL of diluted vehicle and 0.1 mL of IS was added to each volumetric flask. The flasks were diluted to volume with acetone, sealed, and the contents mixed well. This produced single vehicle standards at target concentrations of 0.125, 0.1, 0.075, 0.04, 0.025, and 0.01 mg/mL.

3.4.4 Preparation of Blanks

Vehicle Blank

A single blank was prepared by pipetting 1 mL of diluted vehicle into a 10-mL volumetric flask and diluting to volume with acetone. The flask was sealed and the contents mixed well.

Vehicle Blank with IS

A single blank with IS was prepared by pipetting 1 mL of diluted vehicle and 0.1 mL of IS into a 10-mL volumetric flask and diluting to volume with acetone. The flask was sealed and the contents mixed well.

3.5 Preparation of Formulation Samples For Analysis

3.5.1 Density Determination

For each formulation concentration, a tared 5-mL volumetric flask was filled to volume with the formulation. The weight of the filled flask was recorded and divided by five to obtain the density of the formulation.

3.5.2 Preparation of Formulation Samples

All formulation samples had a stir bar added to the container. The formulations were stirred for at least 5 minutes prior to use.

For each formulation with a concentration less than 100 mg/mL, a 1-mL aliquot was transferred to three previously tared 10-mL volumetric flasks. The weight of the aliquot was recorded. The flasks were diluted to volume with acetone, sealed, and the contents mixed well.

For each formulation with a concentration of 100 mg/mL or greater, a 1-mL aliquot was transferred to three previously tared 25-mL volumetric flasks. The weight of the aliquot was recorded. A 1.5-mL aliquot of corn oil was added to each flask. The flasks were diluted to volume with acetone, sealed, and the contents mixed well.

A 1-mL aliquot of the diluted formulation was transferred to individual 100-mL volumetric flasks. A 1-mL aliquot of IS was added to each flask. The flasks were diluted to volume with acetone, sealed, and the contents mixed well.

3.6 Analysis

Aliquots of each vehicle standard, blank, and diluted sample were transferred into autosampler vials with minimal headspace and the vials were sealed. Single injections were made from each vial using the gas chromatography (GC) instrumental system with flame ionization detection (FID) as shown in Table 4.

Table 4. GC System

Instrument	Agilent 6890 (Santa Clara, CA)
Data System	Thermo Fisher Scientific Atlas, Version 8.2
Column	Restek (Bellefonte, PA),Rtx-5, 30 m × 0.32 mm (ID), 1.0 µm film thickness
Oven Temperature	80°C, hold for 1 minute, increase at 20°C/minute to 200°C, no hold, increase at 10°C/minute to 280°C, hold for 10 minutes
Hydrogen Flow	28 mL/minute
Air Flow Rate	280 mL/minute
Carrier Flow Rate	Helium at 3 mL/minute
Detector Temperature	280°C
Injector Temperature	260°C
Detector Type	FID
Injection Volume	2 µL
Injection Mode	Splitless
Run Time	25 minutes

Representative overlaid chromatograms from a high and low standard, a blank with IS, and a blank are shown in Figure 1.

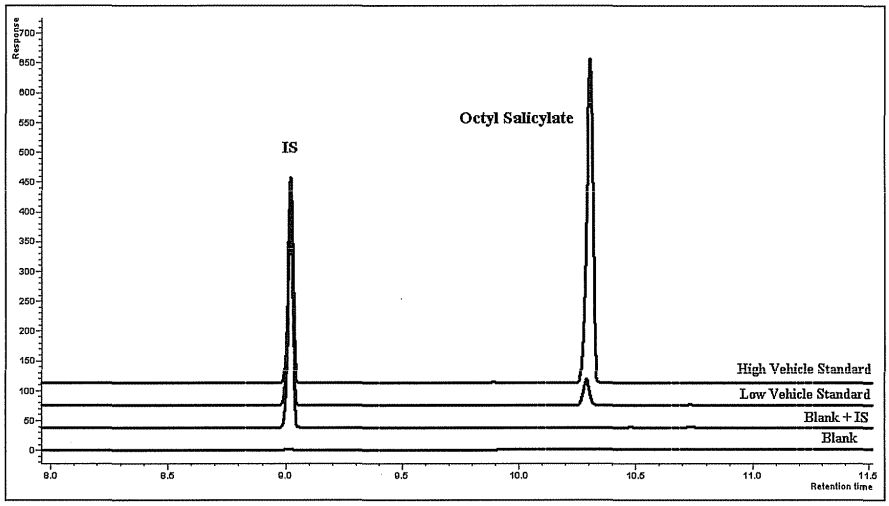

Figure 1. Representative Overlaid Chromatograms

3.7 Calculations

The integration of the octyl salicylate and IS peaks done by the chromatography data system was evaluated and manually adjusted, if necessary, to achieve consistent integration. The response ratio of the octyl salicylate peak area divided by the IS peak area was calculated. A linear regression equation with 1/x weighting was calculated relating the response ratio of the standards to their nominal concentrations. The determined concentration was calculated for each standard and sample using the regression equation, the response ratio for that standard or sample, the sample weight and density, and any dilution factor for the samples. The relative error (RE) for each standard and sample was calculated by subtracting the target concentration from its determined concentration, dividing the difference by the target concentration, and multiplying the result by 100. The average concentration, average RE, standard deviation, and RSD for each formulation location were calculated using the individual values. The grand average concentration, grand RE, grand standard deviation, and grand RSD for each formulation were calculated using the average concentration for each location.

At least one extra significant figure was carried through all calculations to minimize rounding errors, therefore, the summary statistics presented in the tables may not be exactly reproduced using the rounded input values shown.

3.8 Results

The results of the formulation analyses are shown in Table 5. The 0 mg/mL formulations were below the limit of quantitation (BLOQ). The standard curve is shown in Figure 2.

Table 5. Corn Oil Homogeneity Formulation Sample Analysis Results

Target Concentration (Sample ID)	Sample No.	Determined Concentration (mg/mL)	Average Determined Concentration (mg/mL)	s (mg/mL)	RSD	RE	Average RE	Grand Average Determined Concentration (mg/mL)	Grand s (mg/mL)	Grand RSD	Grand RE
64 mg/mL (11-30-1T)	Top A	62.5	62.6	0.3	0.5	-2.3	-2.2	63.1	0.4	0.7	-1.4
	Top B	62.9				-1.7					
	Top C	62.4				-2.5					
64 mg/mL (11-30-1M)	Middle A	63.5	63.3	0.3	0.5	-0.8	-1.1				
	Middle B	63.5				-0.8					
	Middle C	63.0				-1.6					
64 mg/mL (11-30-1B)	Bottom A	63.3	63.4	0.3	0.5	-1.1	-1.0				
	Bottom B	63.7				-0.5					
	Bottom C	63.2				-1.3					
200 mg/mL (11-30-2T)	Top A	194	192	2	1.0	-3.0	-3.8	192	0	0.0	-4.0
	Top B	193				-3.5					
	Top C	190				-5.0					
200 mg/mL (11-30-2M)	Middle A	189	192	3	2.0	-5.5	-4.2				
	Middle B	191				-4.5					
	Middle C	195				-2.5					
200 mg/mL (11-30-2B)	Bottom A	191	192	2	1.0	-4.5	-3.8				
	Bottom B	194				-3.0					
	Bottom C	192				-4.0					

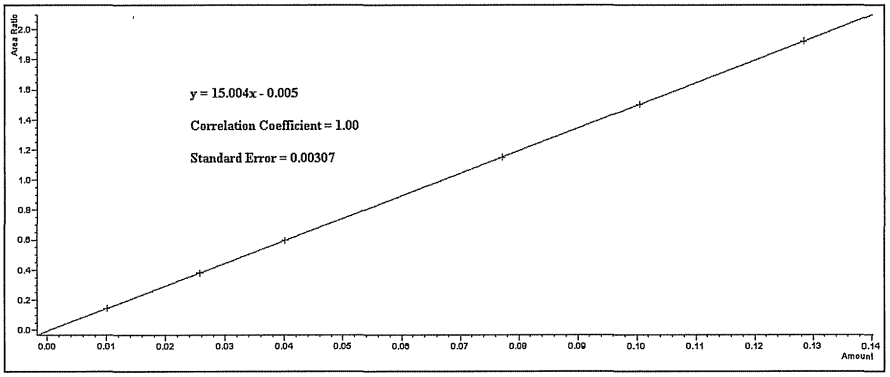

Figure 2. Standard Curve

3.9 Conclusions

The concentrations of all the submitted formulations containing octyl salicylate were within 10 percent of target, the NTP acceptance limit. All formulations were found to

be homogeneous. The 0 mg/mL formulation contained no detectable octyl salicylate. All other quality criteria stated in the SOP were within acceptance limits.

4.0 ACKNOWLEDGMENTS

[redacted] conducted the analysis. [redacted] wrote the report. [redacted] reviewed the analysis raw data for completeness and accuracy.

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BATTELLE-FA

Analytical Chemistry Services for the NTP
NIH Contract No.: HHSN273201000016C
Battelle Project No.: G006623-EEC
NTP ChemTask No.: CHEM11269
CAS No.: 6197-30-4

FORMULATION ANALYSIS OF
2-ETHYLHEXYL 2-CYANO-3,3-DIPHENYLACRYLATE (OCTOCRYLENE)
IN CORN OIL

June 3, 2011

Prepared By:

[redacted]
Study Director

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[redacted]
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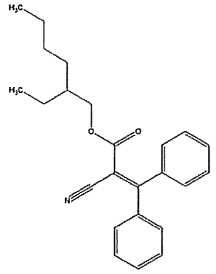
FORMULATION ANALYSIS OF
2-ETHYLHEXYL 2-CYANO-3,3-DIPHENYLACRYLATE (OCTOCRYLENE)
IN CORN OIL

<table>
<tr><td colspan="2">CAS No.: 6197-30-4</td><td>Lot No.: 01697MJ (Sigma-Aldrich, 99.2% purity by GC)</td></tr>
<tr><td colspan="2">Battelle Chemical ID Code: 335</td><td>Samples Analyzed:
<u>Batch</u> <u>Concentration</u>
N135-11-94-5511B 0 mg/mL
11-31-1T 64 mg/mL
11-31-1M 64 mg/mL
11-31-1B 64 mg/mL
11-31-2T 200 mg/mL
11-31-2M 200 mg/mL
11-31-2B 200 mg/mL</td></tr>
<tr><td colspan="2">Battelle Task No.: 16-335-FA-315</td><td>Sample Receipt Date: 5/6/11</td></tr>
<tr><td colspan="2">NTP Task No.: CHEM11269</td><td>Submitter: Integrated Laboratory Systems, Inc. (ILS)</td></tr>
<tr><td colspan="2">Program Supported: TOX</td><td>Study Lab: ILS</td></tr>
<tr><td colspan="2">Analysis Dates: 5/9-5/10/11</td><td>Mix Date: 5/5/11</td></tr>
<tr><td colspan="2">Interim Results Date: 5/12/11</td><td>Receipt Condition: Good</td></tr>
<tr><td colspan="2" rowspan="2">SOPs: CSCSPEC.II-049-00, Standard Operating Procedure for the Analysis of 2-Ethylhexyl 2-Cyano-3,3-Diphenylacrylate (Octocrylene) Formulations in Corn Oil</td><td>Shipping Container: Seven amber glass vials</td></tr>
<tr><td>Storage Conditions (@ Battelle): Refrigerated (~5°C)</td></tr>
<tr><td>Structure
  </td><td>Mol. Wt.

361.48 g/mol</td><td>Mol. Formula

$C_{24}H_{27}NO_2$</td></tr>
</table>

EXECUTIVE SUMMARY

Formulations of octocrylene in corn oil at target concentrations of 0, 64, and 200 mg/mL were prepared by ILS and analyzed by Battelle to determine their concentration and homogeneity prior to administration in support of a TOX study.

The concentrations of all the formulations containing octocrylene were within 10 percent of target, the National Toxicology Program (NTP) acceptance limit. The relative standard deviation (RSD) values were also within the specified acceptance limit. All formulations were found to be homogeneous. The 0 mg/mL formulation contained no detectable octocrylene. All other quality criteria stated in the SOP were within acceptance limits.

QUALITY ASSURANCE STATEMENT

FORMULATION ANALYSIS OF
2-ETHYLHEXYL 2-CYANO-3,3-DIPHENYLACRYLATE (OCTOCRYLENE)
IN CORN OIL

NTP ChemTask No.: CHEM11269
Battelle Project No.: G006623-EEC
Battelle Task No.: 16-335-FA-315

Listed below are the phases and/or procedures performed by Battelle that were reviewed by the Quality Assurance Unit (QAU) during performance of the task described in this report. Adverse findings, if any, were reported to the study director at the time of review.

Critical Phase Inspected	Date Inspected	Date Reported to Study Director and Management
Formulation analysis	5/9/11	5/10/11
Audit study file	5/24/11	5/24/11
Audit final analytical report	5/24/11	5/24/11

This report reflects the procedures and raw data generated in this study.

In addition to the study-specific audits/inspections cited above, routine inspections of the general facilities and equipment were performed by the QAU and reports were submitted to management as follows:

Facility/Equipment	Date Inspected	Date of Report to Management
Chemistry Technical Center Inspection	12/2, 12/15/08	12/2, 12/22/08
	12/16, 12/23/09	12/16, 12/31/09
	12/28, 12/30/10	12/30/10

[redacted] 6-2-11
Quality Assurance Unit Date

COMPLIANCE STATEMENT

This study was conducted in accordance with the Food and Drug Administration's (FDA's) Good Laboratory Practice (GLP) regulations (21 CFR, Part 58), with the exception of archival of study records at the close of the study. These records are gathered, microfiched, and archived periodically for finalized studies for this program.

[redacted] 6-3-11

Study Director Date

Date Study Initiated (Date Protocol Signed): May 5, 2011
Date Study Completed (Date Final Report Signed): June 3, 2011

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0 INTRODUCTION

This report contains:

- A description of the analyses of the formulations for concentration and homogeneity
- Results from the analysis
- Figures
- Conclusions.

This work was performed at Battelle, 505 King Avenue, Columbus, OH 43201, and supports a TOX study.

2.0 FORMULATION SAMPLES

Formulation samples prepared in corn oil (approximately 10 mL each) were received from ILS on May 6, 2011. The samples were formulated on May 5, 2011 with an expiration date of June 17, 2011. They were identified as being from ILS Protocol No. N135-231/232 and had the following concentrations and log numbers.

Table 1. Formulation Samples

Concentration (mg/mL)	ILS Log No.
0	N135-11-94-5511B
64	11-31-1T
64	11-31-1M
64	11-31-1B
200	11-31-2T
200	11-31-2M
200	11-31-2B

All analysis samples that were supplied by ILS in Table 1 were analyzed.

3.0 FORMULATION ANALYSIS FOR CONTENT AND HOMOGENEITY

The formulations were analyzed for octocrylene according to CSCSPEC.II-049-00, "Standard Operating Procedure for the Analysis of 2-Ethylhexyl 2-Cyano-3,3-Diphenylacrylate (Octocrylene) Formulations in Corn Oil." This SOP was based on work originally conducted under the preliminary chemical studies (PCS) task for octocrylene, Battelle Study No. G005430-DYT, NTP ChemTask No. CHEM10882 and the dose formulation developmental (DFD) task for octocrylene in corn oil,

Battelle Study No. G005430-DZY, NTP ChemTask No. CHEM10924. The experimental limit of quantitation (ELOQ) is 0.01 mg/mL, which is the nominal concentration of the lowest standard for this task. This section describes the method, results, and conclusions.

3.1 Preparation of Diluted Vehicle Solution

The diluted vehicle solution was prepared by adding 1 mL of corn oil to a 100-mL volumetric flask and dissolving it in and diluting the flask to volume with acetone. The flask was sealed and the contents mixed well.

3.2 Preparation of Internal Standard (IS)

IS solution was prepared by weighing approximately 125 mg of benzophenone into a 25-mL volumetric flask and dissolving it in and diluting the flask to volume with acetone. The flask was sealed and the contents mixed well.

3.3 Preparation of Standards and Blanks

3.3.1 Stocks

The amounts of octocrylene shown in Table 2 were weighed into individual 50-mL volumetric flasks. The chemical was dissolved in and the flasks diluted to volume with acetone. The flasks were sealed and the contents mixed well.

Table 2. Preparation of Stocks

ID	Target Concentration (mg/mL)	Target Weight (mg)
A	1.25	62.50 ± 2
B	1	50.00 ± 2

3.3.2 Spiking Solutions

The volumes of the A and B indicated in Table 3 were pipetted into individual volumetric flasks. The flasks were diluted to volume with acetone, sealed, and the contents mixed well. A single solution was prepared at all concentrations.

Table 3. Preparation of Spiking Solutions

ID	Target Concentration (mg/mL)	Source	Source Volume (mL)	Final Volume (mL)
C	0.75	A	3	5
D	0.40	B	2	5
E	0.25	A	2	10
F	0.10	B	1	10

3.3.3 Vehicle/Calibration Standards

One (1) mL from each solution A to F was pipetted into individual 10-mL volumetric flasks. One (1) mL of diluted vehicle and 0.1 mL of IS was added to each volumetric flask. The flasks were diluted to volume with acetone, sealed, and the contents mixed well. This produced single vehicle standards at target concentrations of 0.125, 0.1, 0.075, 0.04, 0.025, and 0.01 mg/mL.

3.3.4 Preparation of Blanks

Vehicle Blank

A single blank was prepared by pipetting 1 mL of diluted vehicle into a 10-mL volumetric flask and diluting to volume with acetone. The flask was sealed and the contents mixed well.

Vehicle Blank with IS

A single blank with IS was prepared by pipetting 1 mL of diluted vehicle and 0.1 mL of IS into a 10-mL volumetric flask and diluting to volume with acetone. The flask was sealed and the contents mixed well.

3.4 Preparation of Formulation Samples For Analysis

3.4.1 Density Determination

For each formulation concentration, a tared 5-mL volumetric flask was filled to volume with the formulation. The weight of the filled flask was recorded and divided by five to obtain the density of the formulation.

3.4.2 Preparation of Formulation Samples

All formulation samples had a stir bar added to the container. The formulations were stirred for at least 5 minutes prior to use.

For each formulation sample with a concentration less than 100 mg/mL, a 1-mL aliquot was transferred to three previously tared 10-mL volumetric flasks. The weight of the aliquot was recorded. The flasks were diluted to volume with acetone, sealed, and the contents mixed well.

For each formulation sample with a concentration of 100 mg/mL or greater, a 1-mL aliquot was transferred to three previously tared 25-mL volumetric flasks. The weight of the aliquot was recorded. A 1.5-mL aliquot of corn oil was added to each flask. The flasks were diluted to volume with acetone, sealed, and the contents mixed well.

A 1-mL aliquot of the diluted formulation was transferred to individual 100-mL volumetric flasks. A 1-mL aliquot of IS was added to each flask. The flasks were diluted to volume with acetone, sealed, and the contents mixed well.

3.5 Analysis

Aliquots of each vehicle standard, blank, and diluted sample were transferred into autosampler vials with minimal headspace and the vials were sealed. Single injections were made from each vial using the gas chromatography (GC) instrumental system with flame ionization (FID) detection as shown in Table 4.

Table 4. GC System

Instrument	Agilent 6890 (Santa Clara, CA)
Data System	Thermo Fisher Scientific Atlas, Version 8.2
Column	Restek (Bellefonte, PA), Rtx-5, 30 m × 0.32 mm (ID), 1.0 µm film thickness
Oven Temperature	80°C, hold for 1 minute, increase at 20°C/minute to 200°C, no hold, increase at 10°C/minute to 280°C, hold for 10 minutes
Hydrogen Flow	28 mL/minute
Air Flow Rate	280 mL/minute
Carrier Flow Rate	Helium at 3 mL/minute
Detector Temperature	280°C
Injector Temperature	260°C
Detector Type	FID
Injection Volume	2 µL
Injection Mode	Splitless
Run Time	25 minutes

Representative overlaid chromatograms from a high and low standard, a blank with IS, and a blank are shown in Figure 1.

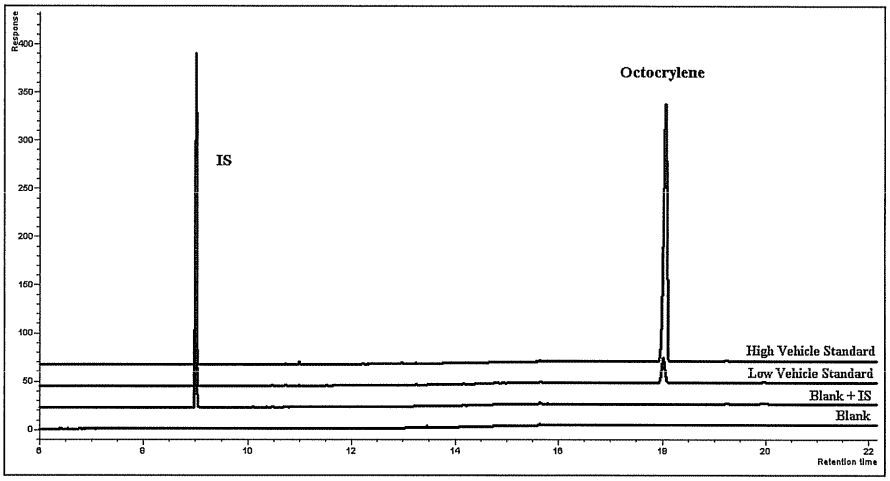


Figure 1. Representative Overlaid Chromatograms

3.6 Calculations

The integration of the octocrylene and IS peaks done by the chromatography data system was evaluated and manually adjusted, if necessary, to achieve consistent integration. The response ratio of the octocrylene peak area divided by the IS peak area was calculated. A linear regression equation with 1/x weighting was calculated relating the response ratio of the standards to their nominal concentrations. The determined concentration was calculated for each standard and sample using the regression equation, the response ratio for that standard or sample, the sample weight and density, and any dilution factor for the samples. The relative error (RE) for each standard and sample was calculated by subtracting the target concentration from its determined concentration, dividing the difference by the target concentration, and multiplying the result by 100. The average concentration, average RE, standard deviation, and RSD for each formulation location were calculated using the individual values. The grand average concentration, grand RE, grand standard deviation, and grand RSD for each formulation were calculated using the average concentration for each location.

At least one extra significant figure was carried through all calculations to minimize rounding errors, therefore, the summary statistics presented in the tables may not be exactly reproduced using the rounded input values shown.

3.7 Results

The results of the formulation analyses are shown in Table 5. The 0 mg/mL formulations were below the limit of quantitation (BLOQ). The standard curve is shown in Figure 2.

Table 5. Corn Oil Homogeneity Formulation Sample Analysis Results

Target Concentration (Sample ID)	Sample No.	Determined Concentration (mg/mL)	Average Determined Concentration (mg/mL)	s (mg/mL)	RSD	RE	Average RE	Grand Average Determined Concentration (mg/mL)	Grand s (mg/mL)	Grand RSD	Grand RE
64 mg/mL (11-31-1T)	Top A	61.0	60.7	0.4	0.7	-4.7	-5.1	60.7	0.8	1.2	-5.2
	Top B	60.9				-4.8					
	Top C	60.3				-5.8					
64 mg/mL (11-31-1M)	Middle A	61.2	61.5	0.3	0.5	-4.4	-4.0				
	Middle B	61.7				-3.6					
	Middle C	61.5				-3.9					
64 mg/mL (11-31-1B)	Bottom A	61.1	60.0	2.0	3.3	-4.5	-6.2				
	Bottom B	61.3				-4.2					
	Bottom C	57.7				-9.8					
200 mg/mL (11-31-2T)	Top A	178	180	3	2.0	-11.0	-10.0	185	6	3.0	-7.5
	Top B	179				-10.5					
	Top C	183				-8.5					
200 mg/mL (11-31-2M)	Middle A	180	185	4	2.0	-10.0	-7.7				
	Middle B	188				-6.0					
	Middle C	186				-7.0					
200 mg/mL (11-31-2B)	Bottom A	192	191	1	0.5	-4.0	-4.3				
	Bottom B	192				-4.0					
	Bottom C	190				-5.0					

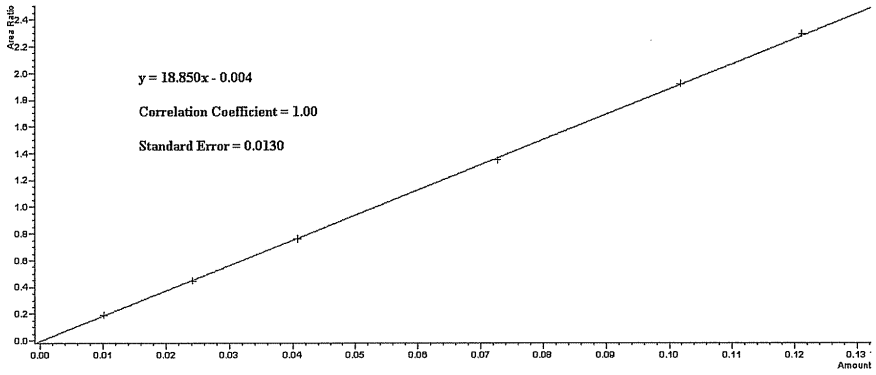


Figure 2. Standard Curve

3.8 Conclusions

The concentrations of all the submitted formulations containing octocrylene were within 10 percent of target, the NTP acceptance limit. The formulations were found

to be homogeneous. The 0 mg/mL formulation contained no detectable octocrylene. All other quality criteria stated in the SOP were within acceptance limits.

4.0 ACKNOWLEDGMENTS

[redacted] conducted the analysis. [redacted] wrote the report. [redacted] reviewed the analysis raw data for completeness and accuracy.

Appendix III:

Dose Times, Volume and Dose Administration

					Dose Administration Day 1 (16 May 2011)			Dose Administration Day 2 (17 May 2011)			Dose Administration Day 3 (18 May 2011)			Dose Administration Day 4 (19 May 2011)			Necropsy Days 4 and 5 (19 May and 20 May 2011)	
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 Death	Time From Last Administration
1	01	Vehicle Control	0	56	12:53	1.2	0	12:46	1.2	0	13:03	1.2	0				12:04	23:01
	02				12:59	1.3	0	12:51	1.3	0	13:12	1.3	0				12:46	23:34
	03				13:05	1.3	0	12:57	1.3	0	13:20	1.3	0				13:25	24:05
	04				13:11	1.3	0	13:02	1.3	0	13:28	1.3	0				14:00	24:32
	05							13:06	1.3	0	13:37	1.3	0	12:58	1.3	0	12:08	23:10
	06							13:11	1.3	0	13:44	1.4	0	13:06	1.4	0	12:44	23:38
	07							13:15	1.4	0	13:52	1.4	0	13:15	1.4	0	13:12	23:57
	08							13:21	1.3	0	13:59	1.3	0	13:21	1.4	0	13:51	24:30
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 Death	Time From Last Administration
2	09	Oxybenzone	63.9	56	12:54	1.2	322.46	12:46	1.2	320.70	13:04	1.2	317.12				12:04	23:00
	10				13:00	1.2	306.84	12:52	1.3	327.82	13:13	1.3	328.08				12:46	23:33
	11				13:06	1.2	310.95	12:57	1.2	314.52	13:21	1.3	328.86				13:26	24:05
	12				13:11	1.3	321.60	13:02	1.3	321.85	13:29	1.3	320.12				14:03	24:34
	13							13:06	1.3	323.73	13:37	1.3	323.73	12:59	1.3	319.50	12:08	23:09
	14							13:11	1.3	313.71	13:45	1.3	313.71	13:07	1.4	332.44	12:44	23:37
	15							13:15	1.3	310.77	13:52	1.4	334.68	13:15	1.4	330.35	13:20	24:05
	16							13:21	1.4	318.70	13:59	1.4	318.70	13:22	1.5	339.77	13:58	24:36
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 Death	Time From Last Administration
3	17	Oxybenzone	211	56	12:55	1.2	1056.76	12:47	1.2	1064.76	13:05	1.2	1056.76				12:11	23:06
	18				13:01	1.2	1035.58	12:52	1.2	1035.16	13:13	1.2	1041.98				12:54	23:41
	19				13:07	1.2	1022.62	12:58	1.2	1044.99	13:22	1.2	1036.43				13:34	24:12
	20				13:12	1.3	1080.77	13:02	1.3	1078.22	13:30	1.3	1082.48				14:10	24:40
	21							13:07	1.3	1057.85	13:38	1.3	1086.34	13:00	1.3	1089.36	12:15	23:15
	22							13:11	1.3	1025.04	13:46	1.3	1055.00	13:08	1.3	1042.17	12:51	23:43
	23							13:16	1.3	1025.42	13:53	1.3	1061.12	13:16	1.3	1064.83	13:23	24:07
	24							13:21	1.3	1016.68	14:00	1.3	1003.66	13:23	1.4	1101.83	13:58	24:35

					Dose Administration Day 1 (16 May 2011)			Dose Administration Day 2 (17 May 2011)			Dose Administration Day 3 (18 May 2011)			Dose Administration Day 4 (19 May 2011)			Necropsy Days 4 and 5 (19 May and 20 May 2011)	
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 Death	Time From Last Administration
4	25	Octylmethoxycinnamate	65.2	56	12:56	1.2	314.09	12:47	1.2	315.48	13:06	1.3	332.78				12:13	23:07
	26				13:01	1.3	339.99	12:53	1.3	333.83	13:14	1.3	332.00				12:54	23:40
	27				13:08	1.3	339.04	12:58	1.3	333.70	13:22	1.3	331.09				13:34	24:12
	28				13:12	1.3	327.26	13:03	1.3	322.16	13:31	1.3	318.05				14:09	24:38
	29							13:07	1.3	320.57	13:39	1.4	345.23	13:00	1.4	336.95	12:16	23:16
	30							13:12	1.3	318.77	13:47	1.3	318.77	13:09	1.4	338.70	12:52	23:43
	31							13:17	1.4	336.83	13:54	1.3	312.77	13:17	1.3	317.93	13:29	24:12
	32							13:22	1.4	329.65	14:01	1.4	329.65	13:23	1.4	332.17	14:07	24:44
Group No.:	Animal No.	Test Substance/Dose Level	Actual Dose Concentration (mg/ml)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
5	33	Octylmethoxycinnamate	202.9	56	12:56	1.2	1019.60	12:48	1.2	1014.08	13:07	1.2	1009.45				12:23	23:16
	34				13:02	1.2	993.39	12:54	1.2	988.15	13:15	1.3	1033.18				13:02	23:47
	35				13:08	1.2	979.40	12:59	1.3	1040.10	13:23	1.3	1029.95				13:41	24:18
	36				13:13	1.3	1006.76	13:03	1.3	1004.46	13:32	1.3	1038.87				14:16	24:44
	37							13:08	1.3	1004.46	13:40	1.3	1004.46	13:02	1.3	1093.57	12:22	23:20
	38							13:12	1.3	998.75	13:47	1.3	998.75	13:11	1.4	1112.65	12:59	23:48
	39							13:18	1.4	1045.88	13:55	1.3	971.17	13:17	1.4	1109.18	13:29	24:12
	40							13:22	1.4	1004.46	14:02	1.4	1004.46	13:24	1.4	1118.79	14:08	24:44
Group No.:	Animal No.	Test Substance/Dose Level	Actual Dose Concentration (mg/ml)	Age of Animal (PND)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Administration (Hour:Minute)	Volume Administered (mL)	Actual Dose Received (mg/kg)	Time of Death	Time From Last Administration
6	41	Octylsalate	63.1	56	12:57	1.2	310.07	12:48	1.2	320.98	13:07	1.2	319.09				12:22	23:15
	42				13:02	1.3	327.86	12:54	1.2	308.81	13:16	1.3	323.85				13:02	23:46
	43				13:08	1.3	322.07	12:59	1.2	309.82	13:24	1.2	307.43				13:41	24:17
	44				13:13	1.3	324.10	13:04	1.2	304.46	13:32	1.3	320.43				14:16	24:44
	45							13:08	1.3	305.17	13:41	1.3	305.17	13:02	1.3	313.81	12:23	23:21
	46							13:13	1.3	310.96	13:48	1.3	310.96	13:11	1.4	328.89	12:59	23:48
	47							13:18	1.4	326.82	13:55	1.4	326.82	13:18	1.3	302.36	13:37	24:19
	48							13:23	1.4	318.69	14:02	1.4	318.69	13:24	1.4	315.95	14:15	24:51

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 Death	Time From Last Administration
7	49	Octylsalate	192	56	12:58	1.2	957.61	12:49	1.2	983.77	13:08	1.1	936.17				12:30	23:22
	50				13:03	1.3	994.42	12:55	1.2	929.78	13:17	1.2	957.61				13:09	23:52
	51				13:09	1.3	988.12	13:00	1.3	979.21	13:25	1.2	941.18				13:49	24:24
	52				13:14	1.3	982.29	13:04	1.2	937.73	13:33	1.2	982.94				14:22	24:49
	53							13:09	1.3	950.13	13:41	1.3	950.13	13:03	1.2	909.95	12:29	23:26
	54							13:13	1.3	942.95	13:49	1.3	942.95	13:12	1.3	955.96	8:59	19:47
	55							13:19	1.3	938.35	13:56	1.3	938.35	13:19	1.3	972.72	13:37	24:18
	56							13:23	1.3	927.54	14:03	1.3	927.54	13:25	1.3	970.45	8:57	19:32
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 Death**	**Time From Last Administration**
8	57	Octocrylene	60.7	56	12:58	1.2	301.86	12:50	1.2	302.12	13:09	1.2	295.38				12:30	23:21
	58				13:03	1.2	296.10	12:55	1.3	313.88	13:18	1.3	310.30				13:09	23:51
	59				13:10	1.2	294.78	13:00	1.2	293.35	13:25	1.2	291.48				13:49	24:24
	60				13:14	1.2	292.30	13:04	1.3	309.33	13:34	1.3	306.09				14:03	24:29
	61							13:09	1.3	300.27	13:42	1.3	300.27	13:04	1.4	316.85	12:29	23:25
	62							13:14	1.3	303.27	13:49	1.3	303.27	13:13	1.4	320.80	13:06	23:53
	63							13:19	1.3	299.58	13:57	1.3	299.58	13:19	1.4	320.68	13:45	24:26
	64							13:24	1.4	309.92	14:03	1.4	309.92	13:26	1.4	308.57	14:15	24:49
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 Death**	**Time From Last Administration**
9	65	Octocrylene	185	56	12:59	1.2	895.16	12:50	1.2	903.17	13:10	1.2	902.44				12:30	23:20
	66				13:04	1.2	898.79	12:56	1.3	960.46	13:19	1.3	952.48				13:17	23:58
	67				13:10	1.3	936.53	13:01	1.3	955.88	13:27	1.3	938.72				13:56	24:29
	68				13:15	1.2	890.14	13:05	1.3	946.85	13:35	1.3	920.75				14:29	24:54
	69							13:10	1.3	921.10	13:43	1.3	921.10	13:04	1.4	978.10	12:29	23:25
	70							13:14	1.3	904.14	13:50	1.4	973.68	13:13	1.4	949.41	13:06	23:53
	71							13:20	1.4	950.81	13:58	1.4	950.81	13:20	1.4	958.55	13:45	24:25
	72							13:24	1.4	948.72	14:04	1.4	948.72	13:26	1.4	957.49	14:15	24:49

					Dose Administration Day 1 (16 May 2011)		Dose Administration Day 2 (17 May 2011)		Dose Administration Day 3 (18 May 2011)		Dose Administration Day 4 (19 May 2011)		Necropsy Days 4 and 5 (19 May and 20 May 2011)	
Group No.:	Animal No.	Test Substance/Dose Level	Dose Concentration (mg/ml)	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 Death	Time From Last Administration
10	73	17α-ethinyl estradiol	0.1		12:59	1.2	12:51	1.2	13:11	1.2			12:38	23:27
	74				13:05	1.2	12:56	1.2	13:20	1.2			13:17	23:57
	75				13:10	1.3	13:01	1.2	13:27	1.2			13:56	24:29
	76				13:15	1.3	13:06	1.2	13:35	1.2			14:28	24:53
	77						13:10	1.3	13:43	1.4	13:05	1.3	12:36	23:31
	78						13:15	1.4	13:51	1.3	13:14	1.3	13:12	23:58
	79						13:20	1.4	13:58	1.4	13:21	1.4	13:51	24:30
	80						13:25	1.4	14:05	1.3	13:27	1.3	14:22	24:55

Appendix IV:

Clinical Observations

				Day 1		Day 2		Day 3		Terminal
Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**
1	01	F	Corn Oil Control	normal	normal	normal	normal	normal	normal	normal
1	02	F		normal	normal	normal	normal	normal	normal	normal
1	03	F		normal	normal	normal	normal	normal	normal	normal
1	04	F		normal	normal	normal	normal	normal	normal	normal
1	05	F		normal	normal	normal	normal	normal	normal	normal
1	06	F		normal	normal	normal	normal	normal	normal	normal
1	07	F		normal	normal	normal	normal	normal	normal	normal
1	08	F		normal	normal	normal	normal	normal	normal	normal

Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**
2	09	F	Oxybenzone (320mg/kg)	normal	normal	normal	normal	normal	normal	normal
2	10	F		normal	normal	normal	normal	normal	normal	normal
2	11	F		normal	normal	normal	normal	normal	normal	normal
2	12	F		normal	normal	normal	normal	normal	normal	normal
2	13	F		normal	normal	normal	normal	normal	normal	normal
2	14	F		normal	normal	normal	normal	normal	normal	normal
2	15	F		normal	normal	normal	normal	normal	normal	normal
2	16	F		normal	normal	normal	normal	normal	normal	normal

Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**
3	17	F	Oxybenzone (1000mg/kg)	normal	normal	normal	normal	normal	normal	normal
3	18	F		normal	normal	normal	normal	normal	normal	normal
3	19	F		normal	normal	normal	normal	normal	normal	normal
3	20	F		normal	normal	normal	normal	normal	normal	normal
3	21	F		normal	normal	normal	normal	normal	normal	normal
3	22	F		normal	normal	normal	normal	normal	normal	normal
3	23	F		normal	normal	normal	normal	normal	normal	normal
3	24	F		normal	normal	normal	normal	normal	normal	normal

				Day 1		Day 2		Day 3		Terminal
Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Clinical**	**PD Clinical**	**Clinical**	**PD Clinical**	**Clinical**	**PD Clinical**	**Clinical**
4	25	F	Octylmethoxycinnamate (320 mg/kg)	normal	normal	normal	normal	normal	normal	normal
4	26	F		normal	normal	normal	normal	normal	normal	normal
4	27	F		normal	normal	normal	normal	normal	normal	normal
4	28	F		normal	normal	normal	normal	normal	normal	normal
4	29	F		normal	normal	normal	normal	normal	normal	normal
4	30	F		normal	normal	normal	normal	normal	normal	normal
4	31	F		normal	normal	normal	normal	normal	normal	normal
4	32	F		normal	normal	normal	normal	normal	normal	normal
Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**
5	33	F	Octylmethoxycinnamate (1000 mg/kg)	normal	normal	normal	normal	normal	normal	normal
5	34	F		normal	normal	normal	normal	normal	normal	normal
5	35	F		normal	normal	normal	normal	normal	normal	normal
5	36	F		normal	normal	normal	normal	normal	normal	normal
5	37	F		normal	normal	normal	normal	normal	normal	normal
5	38	F		normal	normal	normal	normal	normal	normal	normal
5	39	F		normal	normal	normal	normal	normal	normal	normal
5	40	F		normal	normal	normal	normal	normal	normal	normal

Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**
6	41	F	Octylsalate (320mg/kg)	normal	normal	normal	normal	normal	normal	normal
6	42	F		normal	normal	normal	normal	normal	normal	normal
6	43	F		normal	normal	normal	normal	normal	normal	normal
6	44	F		normal	normal	normal	normal	normal	normal	normal
6	45	F		normal	normal	normal	normal	normal	normal	normal
6	46	F		normal	normal	normal	normal	normal	normal	normal
6	47	F		normal	normal	normal	normal	normal	normal	normal
6	48	F		normal	normal	normal	normal	normal	normal	normal

Group No.:	Animal No.	Sex	Treatment/Dose Level	Clinical Observation	PD Clinical Observation	Clinical Observation	PD Clinical Observation	Clinical Observation	PD Clinical Observation	Clinical Observation
7	49	F	Octylsalate (1000mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	50	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	51	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	52	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	53	F		Normal	Normal	Normal	Normal	Normal	Normal	uncoordinated movement, hunched posture
7	54	F		Normal	Normal	Normal	Normal	Normal	Normal	*
7	55	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal
7	56	F		Normal	Normal	Normal	Normal	Normal	Normal	*

*Animal died prior to scheduled necropsy

Group No.:	Animal No.	Sex	Treatment/Dose Level	Clinical Observation	PD Clinical Observation	Clinical Observation	PD Clinical Observation	Clinical Observation	PD Clinical Observation	Clinical Observation
8	57	F	Octocrylene (320mg/kg)	Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	58	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	59	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	60	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	61	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	62	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	63	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal
8	64	F		Normal	Normal	Normal	Normal	Normal	Normal	Normal

				Day 1		Day 2		Day 3		Terminal
Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Clinical**	**PD Clinical**	**Clinical**	**PD Clinical**	**Clinical**	**PD Clinical**	**Clinical**
9	65	F	Octocrylene (1000mg/kg)	normal	normal	normal	normal	normal	normal	normal
9	66	F		normal	normal	normal	normal	normal	normal	normal
9	67	F		normal	normal	normal	normal	normal	normal	normal
9	68	F		normal	normal	normal	normal	normal	normal	normal
9	69	F		normal	normal	normal	normal	normal	normal	normal
9	70	F		normal	normal	normal	normal	normal	normal	normal
9	71	F		normal	normal	normal	normal	normal	normal	normal
9	72	F		normal	normal	normal	normal	normal	normal	normal

Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**	**PD Clinical Observation**	**Clinical Observation**
10	73	F	17α -ethinyl estradiol (0.1mg/kg)	normal	normal	normal	normal	normal	normal	normal
10	74	F		normal	normal	normal	normal	normal	normal	normal
10	75	F		normal	normal	normal	normal	normal	normal	normal
10	76	F		normal	normal	normal	normal	normal	normal	normal
10	77	F		normal	normal	normal	normal	normal	normal	normal
10	78	F		normal	normal	normal	normal	normal	normal	normal
10	79	F		normal	normal	normal	normal	normal	normal	normal
10	80	F		normal	normal	normal	normal	normal	normal	normal

Appendix V:

Body Weight Data

				Day 1	Day 2	Day 3	Terminal		
Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**% of Control (PND 35)**	**Body Weight Gain (g)**
1	01	F	Corn Oil Control	232.3	238.9	243.1	249.4		17.1
1	02	F		251.1	255.0	258.5	263.5		12.4
1	03	F		252.2	256.6	261.9	266.3		14.1
1	04	F		261.2	264.0	263.7	269.1		7.9
1	05	F		257.1	257.1	259.9	264.3		7.2
1	06	F		268.8	268.8	271.8	276.7		7.9
1	07	F		273.3	273.3	277.6	271.5		-1.8
1	08	F		265.5	265.5	263.4	284.2		18.7
			Mean	**257.7**	**259.9**	**262.5**	**268.1**		**10.4**
			St. dev.	**12.9**	**10.6**	**10.1**	**10.2**		**6.6**
			Count	**8**	**8**	**8**	**8**		**8**

Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**% of Control (PND 35)**	**Body Weight Gain (g)**
2	09	F	Oxybenzone (320mg/kg)	237.8	239.1	241.8	247.0	92.1	9.2
2	10	F		249.9	253.4	253.2	264.5	98.6	14.6
2	11	F		246.6	243.8	252.6	262.2	97.8	15.6
2	12	F		258.3	258.1	259.5	267.0	99.6	8.7
2	13	F		256.6	256.6	260	264.6	98.7	8.0
2	14	F		264.8	264.8	269.1	277.3	103.4	12.5
2	15	F		267.3	267.3	270.8	276.3	103.0	9.0
2	16	F		280.7	280.7	282.1	291.5	108.7	10.8
			Mean	**257.8**	**258.0**	**261.1**	**268.8**	**100.3**	**11.1**
			St. dev.	**13.4**	**13.3**	**12.6**	**13.1**	**4.9**	**2.9**
			Count	**8**	**8**	**8**	**8**	**8**	**8**

Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**% of Control (PND 35)**	**Body Weight Gain (g)**
3	17	F	Oxybenzone (1000mg/kg)	239.6	237.8	239.6	244.6	91.2	5.0
3	18	F		244.5	244.6	243.0	249.6	93.1	5.1
3	19	F		247.6	242.3	244.3	245.0	91.4	-2.6
3	20	F		253.8	254.4	253.4	253.3	94.5	-0.5
3	21	F		259.3	259.3	251.8	252.5	94.2	-6.8
3	22	F		267.6	267.6	263.2	260.0	97.0	-7.6
3	23	F		267.5	267.5	257.6	258.5	96.4	-9.0
3	24	F		269.8	269.8	268.1	273.3	101.9	3.5
			Mean	**256.2**	**255.4**	**252.6**	**254.6**	**95.0**	**-1.6**
			St. dev.	**11.6**	**12.6**	**10.1**	**9.4**	**3.5**	**5.8**
			Count	**8**	**8**	**8**	**8**	**8**	**8**

				Day 1	Day 2	Day 3	Terminal		
Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**% of Control (PND 35)**	**Body Weight Gain (g)**
4	25	F	Octylmethoxycinnamate (320 mg/kg)	249.1	248.0	254.7	260.3	97.1	11.2
4	26	F		249.3	253.9	255.3	264.7	98.7	15.4
4	27	F		250.0	254.0	256.0	263.3	98.2	13.3
4	28	F		259.0	263.1	266.5	272.1	101.5	13.1
4	29	F		264.4	264.4	270.9	277.6	103.5	13.2
4	30	F		265.9	265.9	269.5	282.1	105.2	16.2
4	31	F		271.0	271.0	266.6	267.8	99.9	-3.2
4	32	F		276.9	276.9	274.8	284.4	106.1	7.5
			Mean	**260.7**	**262.2**	**264.3**	**271.5**	**101.3**	**10.8**
			St. dev.	**10.6**	**9.6**	**7.9**	**9.0**	**3.4**	**6.3**
			Count	**8**	**8**	**8**	**8**	**8**	**8**

Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**% of Control (PND 35)**	**Body Weight Gain (g)**
5	33	F	Octylmethoxycinnamate (1000 mg/kg)	238.8	240.1	241.2	243.2	90.7	4.4
5	34	F		245.1	246.4	255.3	258.2	96.3	13.1
5	35	F		248.6	253.6	256.1	259.1	96.6	10.5
5	36	F		262.0	262.6	253.9	259.7	96.9	-2.3
5	37	F		262.6	262.6	267.5	268.8	100.3	6.2
5	38	F		264.1	264.1	267.7	276.3	103.0	12.2
5	39	F		271.6	271.6	265.3	272.6	101.7	1.0
5	40	F		282.8	282.8	283.6	280.2	104.5	-2.6
			Mean	**259.5**	**260.5**	**261.3**	**264.8**	**98.7**	**5.3**
			St. dev.	**14.5**	**13.7**	**12.6**	**12.0**	**4.5**	**6.3**
			Count	**8**	**8**	**8**	**8**	**8**	**8**

Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**% of Control (PND 35)**	**Body Weight Gain (g)**
6	41	F	Octylsalate (320mg/kg)	244.2	235.9	237.3	242.0	90.3	-2.2
6	42	F		250.2	245.2	253.3	253.5	94.5	3.3
6	43	F		254.7	244.4	246.3	246.2	91.8	-8.5
6	44	F		253.1	248.7	256.0	258.7	96.5	5.6
6	45	F		268.8	268.8	261.4	268.3	100.1	-0.5
6	46	F		263.8	263.8	268.6	274.2	102.3	10.4
6	47	F		270.3	270.3	271.3	268.5	100.1	-1.8
6	48	F		277.2	277.2	279.6	277.4	103.5	0.2
			Mean	**260.3**	**256.8**	**259.2**	**261.1**	**97.4**	**0.8**
			St. dev.	**11.4**	**15.0**	**13.9**	**13.1**	**4.9**	**5.7**
			Count	**8**	**8**	**8**	**8**	**8**	**8**

Group No.:	Animal No.	Sex	Treatment/Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	% of Control (PND 35)	Body Weight Gain (g)
7	49	F	Octylsalate (1000mg/kg)	240.6	234.2	225.6	218.3	81.4	-22.3
7	50	F		251.0	247.8	240.6	232.6	86.8	-18.4
7	51	F		252.6	254.9	244.8	233.6	87.1	-19.0
7	52	F		254.1	245.7	234.4	232.2	86.6	-21.9
7	53	F		262.7	262.7	253.2	247.3	92.2	-15.4
7	54	F		264.7	264.7	261.1	*		
7	55	F		266.0	266.0	256.6	266.7	99.5	0.7
7	56	F		269.1	269.1	257.2	*		
			Mean	**257.6**	**255.6**	**246.7**	**238.5**	**88.9**	**-16.1**
			St. dev.	**9.6**	**12.2**	**12.5**	**16.6**	**6.2**	**8.6**
			Count	**8**	**8**	**8**	**6**	**6**	**6**
			*Animal died prior to scheduled necropsy						

Group No.:	Animal No.	Sex	Treatment/Dose Level	Body Weight (g)	Body Weight (g)	Body Weight (g)	Body Weight (g)	% of Control (PND 35)	Body Weight Gain (g)
8	57	F	Octocrylene (320mg/kg)	241.3	241.1	246.6	251.0	93.6	9.7
8	58	F		246.0	251.4	254.3	262.7	98.0	16.7
8	59	F		247.1	248.3	249.9	257.0	95.9	9.9
8	60	F		249.2	255.1	257.8	263.5	98.3	14.3
8	61	F		262.8	262.8	268.2	274.5	102.4	11.7
8	62	F		260.2	260.2	264.9	274.2	102.3	14.0
8	63	F		263.4	263.4	265.0	271.7	101.3	8.3
8	64	F		274.2	274.2	275.4	285.8	106.6	11.6
			Mean	**255.5**	**257.1**	**260.3**	**267.6**	**99.8**	**12.0**
			St. dev.	**11.3**	**10.3**	**9.8**	**11.1**	**4.2**	**2.8**
			Count	**8**	**8**	**8**	**8**	**8**	**8**

				Day 1	Day 2	Day 3	Terminal		
Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**% of Control (PND 35)**	**Body Weight Gain (g)**
9	65	F	Octocrylene (1000mg/kg)	248.0	245.8	246.0	250.3	93.4	2.3
9	66	F		247.0	250.4	252.5	257.4	96.0	10.4
9	67	F		256.8	251.6	256.2	261.8	97.6	5.0
9	68	F		249.4	254.0	261.2	268.7	100.2	19.3
9	69	F		261.1	261.1	264.8	271.1	101.1	10.0
9	70	F		266.0	266.0	272.8	278.8	104.0	12.8
9	71	F		272.4	272.4	270.2	275.5	102.8	3.1
9	72	F		273.0	273.0	270.5	275.0	102.6	2.0
			Mean	**259.2**	**259.3**	**261.8**	**267.3**	**99.7**	**8.1**
			St. dev.	**10.6**	**10.4**	**9.6**	**9.9**	**3.7**	**6.1**
			Count	**8**	**8**	**8**	**8**	**8**	**8**

Group No.:	**Animal No.**	**Sex**	**Treatment/Dose Level**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**Body Weight (g)**	**% of Control (PND 35)**	**Body Weight Gain (g)**
10	73	F	17α -ethinyl estradiol (0.1 mg/kg)	247.0	238.4	237.5	235.6	87.9	-11.4
10	74	F		245.3	244.3	243.0	239.4	89.3	-5.9
10	75	F		252.3	249.3	241.9	242.2	90.3	-10.1
10	76	F		253.0	249.1	249.9	243.2	90.7	-9.8
10	77	F		268.2	268.2	271.2	265.8	99.1	-2.4
10	78	F		271.8	271.8	266.2	263.7	98.3	-8.1
10	79	F		272.9	272.9	273.5	277.5	103.5	4.6
10	80	F		276.9	276.9	266.0	264.4	98.6	-12.5
			Mean	**260.9**	**258.9**	**256.2**	**254.0**	**94.7**	**-7.0**
			St. dev.	**12.8**	**15.1**	**14.6**	**15.6**	**5.8**	**5.7**
			Count	**8**	**8**	**8**	**8**	**8**	**8**

Appendix VI:

Tissue Weight Data

Group No.:	Animal No.:	Sex	Treatment/Dose Level	Terminal Body Weight (g)	Abs. Uterine Weight, wet (g)	Abs. Uterine Weight, blotted (g)	Relative Uterine Weight, blotted (g)
1	01	F	Corn Oil Control	249.4	0.0982	0.0937	0.0376
1	02	F		263.5	0.1069	0.1013	0.0384
1	03	F		266.3	0.0890	0.0815	0.0306
1	04	F		269.1	0.1029	0.0928	0.0345
1	05	F		264.5	0.0926	0.0822	0.0311
1	06	F		280.3	0.1048	0.0958	0.0342
1	07	F		270.3	0.0981	0.0899	0.0333
1	08	F		285.1	0.0617	0.0570	0.0200
			Mean	**268.6**	**0.0943**	**0.0868**	**0.0325**
			St. dev.	**10.9**	**0.0145**	**0.0137**	**0.0057**
			CV	**4.1**	**15.3523**	**15.8355**	**17.6788**

Group No.:	Animal No.:	Sex	Treatment/Dose Level	Terminal Body Weight (g)	Abs. Uterine Weight, wet (g)	Abs. Uterine Weight, blotted (g)
2	09	F	Oxybenzone (320 mg/kg)	247.0	0.0998	0.0939
2	10	F		264.5	0.9340	0.0870
2	11	F		262.2	0.0844	0.0798
2	12	F		267.0	0.0974	0.0894
2	13	F		267.3	0.0893	0.0821
2	14	F		274.5	0.0808	0.0735
2	15	F		279.4	0.1174	0.1083
2	16	F		288.2	0.0921	0.0856
			Mean	**268.8**	**0.1994**	**0.0875**
			St. dev.	**12.3**	**0.2970**	**0.0105**
			CV	**4.6**	**148.9648**	**11.9581**

Group No.:	Animal No.:	Sex	Treatment/Dose Level	Terminal Body Weight (g)	Abs. Uterine Weight, wet (g)	Abs. Uterine Weight, blotted (g)
3	17	F	Oxybenzone (1000 mg/kg)	244.6	0.1079	0.1020
3	18	F		249.6	0.1014	0.0947
3	19	F		245.0	0.1194	0.1119
3	20	F		253.3	0.0804	0.0730
3	21	F		259.8	0.1318	0.1223
3	22	F		268.4	0.1185	0.1075
3	23	F		264.9	0.0908	0.0855
3	24	F		273.8	0.0802	0.0737
			Mean	**257.4**	**0.1038**	**0.0963**
			St. dev.	**11.0**	**0.0190**	**0.0179**
			CV	**4.3**	**18.3498**	**18.6130**

Group No.:	Animal No.:	Sex	Treatment/Dose Level	Terminal Body Weight (g)	Abs. Uterine Weight, wet (g)	Abs. Uterine Weight, blotted (g)
4	25	F	Octylmethoxycinnamate (320 mg/kg)	260.3	0.0846	0.0800
4	26	F		264.7	0.0747	0.0681
4	27	F		263.3	0.1013	0.0932
4	28	F		272.1	0.0995	0.0945
4	29	F		282.4	0.0741	0.0659
4	30	F		277.0	0.0838	0.0752
4	31	F		275.6	0.0977	0.0916
4	32	F		285.7	0.0973	0.0892
			Mean	**272.6**	**0.0891**	**0.0822**
			St. dev.	**9.2**	**0.0112**	**0.0115**
			CV	**3.4**	**12.5725**	**14.0050**

Group No.:	Animal No.:	Sex	Treatment/Dose Level	Terminal Body Weight (g)	Abs. Uterine Weight, wet (g)	Abs. Uterine Weight, blotted (g)
5	33	F	Octylmethoxycinnamate (1000 mg/kg)	243.2	0.0985	0.0905
5	34	F		258.2	0.1087	0.1029
5	35	F		259.1	0.1001	0.0940
5	36	F		259.7	0.0911	0.0830
5	37	F		268.8	0.0928	0.0865
5	38	F		276.9	0.0996	0.0918
5	39	F		277.0	0.0861	0.0808
5	40	F		284.6	0.0779	0.0705
			Mean	**265.9**	**0.0944**	**0.0875**
			St. dev.	**13.4**	**0.0095**	**0.0097**
			CV	**5.0**	**10.0979**	**11.1144**

Group No.:	Animal No.:	Sex	Treatment/Dose Level	Terminal Body Weight (g)	Abs. Uterine Weight, wet (g)	Abs. Uterine Weight, blotted (g)
6	41	F	Octylsalate (320 mg/kg)	242.0	0.0738	0.0675
6	42	F		253.5	0.0831	0.0768
6	43	F		246.2	0.0938	0.0895
6	44	F		258.7	0.1052	0.0990
6	45	F		268.1	0.0875	0.0798
6	46	F		268.5	0.0912	0.0861
6	47	F		260.4	0.0919	0.0850
6	48	F		275.8	0.1165	0.1086
			Mean	**259.2**	**0.0929**	**0.0865**
			St. dev.	**11.6**	**0.0131**	**0.0129**
			CV	**4.5**	**14.1149**	**14.8502**

Group No.:	Animal No.:	Sex	Treatment/Dose Level	Terminal Body Weight (g)	Abs. Uterine Weight, wet (g)	Abs. Uterine Weight, blotted (g)
7	49	F	Octylsalate (1000 mg/kg)	218.3	0.1065	0.0976
7	50	F		232.6	0.0914	0.0843
7	51	F		233.6	0.0928	0.0855
7	52	F		232.2	0.0816	0.0745
7	53	F		230.3	0.1014	0.0946
7	54	F		*	*	*
7	55	F		251.3	0.0935	0.0854
7	56	F		*	*	*
			Mean	**233.1**	**0.0945**	**0.0870**
			St. dev.	**10.6**	**0.0086**	**0.0082**
			CV	**4.5**	**9.1184**	**9.4588**
	***Animal died prior to scheduled necropsy**					

Group No.:	Animal No.:	Sex	Treatment/Dose Level	Terminal Body Weight (g)	Abs. Uterine Weight, wet (g)	Abs. Uterine Weight, blotted (g)
8	57	F	Octocrylene (320 mg/kg)	251.0	0.0878	0.0820
8	58	F		262.7	0.0851	0.0768
8	59	F		257.0	0.0985	0.0928
8	60	F		263.5	0.0972	0.0899
8	61	F		278.3	0.0727	0.0659
8	62	F		278.1	0.0733	0.0672
8	63	F		277.0	0.0851	0.0782
8	64	F		286.4	0.0921	0.0874
			Mean	**269.3**	**0.0865**	**0.0800**
			St. dev.	**12.4**	**0.0097**	**0.0100**
			CV	**4.6**	**11.2299**	**12.4669**

Group No.:	Animal No.:	Sex	Treatment/Dose Level	Terminal Body Weight (g)	Abs. Uterine Weight, wet (g)	Abs. Uterine Weight, blotted (g)
9	65	F	Octocrylene (1000 mg/kg)	250.3	0.0891	0.0839
9	66	F		257.4	0.0908	0.0860
9	67	F		261.8	0.0873	0.0790
9	68	F		268.7	0.0863	0.0821
9	69	F		274.2	0.0843	0.0786
9	70	F		279.4	0.0918	0.0846
9	71	F		282.6	0.1030	0.0957
9	72	F		275.3	0.0987	0.0921
			Mean	**268.7**	**0.0914**	**0.0853**
			St. dev.	**11.3**	**0.0064**	**0.0060**
			CV	**4.2**	**7.0033**	**7.0401**

Group No.:	Animal No.:	Sex	Treatment/Dose Level	Terminal Body Weight (g)	Abs. Uterine Weight, wet (g)	Abs. Uterine Weight, blotted (g)
10	73	F	17α-Ethinyl Estradiol (0.1 mg/kg)	235.6	0.3050	0.1940
10	74	F		239.4	0.2392	0.2068
10	75	F		242.2	0.1767	0.1619
10	76	F		243.2	0.2495	0.2211
10	77	F		265.5	0.3373	0.2453
10	78	F		259.6	0.2462	0.1928
10	79	F		268.3	0.2386	0.2175
10	80	F		257.6	0.4241	0.2416
			Mean	**251.4**	**0.2771**	**0.2101**
			St. dev.	**12.7**	**0.0763**	**0.0275**
			CV	**5.1**	**27.5292**	**13.0948**

Appendix VII:

Study Protocol

Study Title
The Uterotrophic Assay (OPPTS 890.1600) with Oxybenzone, Octylmethoxycinnamate, Octylsalate, and Octocrylene

ILS Project-Study Number
N135-231

Performing Laboratory
Integrated Laboratory Systems, Inc.
635 Keystone Park Drive, Suite 100
Durham, NC27713

Sponsor
National Institutes of Environmental Health
P.O. Box 12233
Research Triangle Park, NC27709

Study Protocol Approval

[redacted]

Chief Toxicology Branch
National Toxicology Program, NIEHS

5/5/11
Date

[redacted]

Contract Office Technical Representative
National Toxicology Program, NIEHS

5/5/11
Date

[redacted]

Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

5/6/11
Date

[redacted]

Principal Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

5/9/11
Date

TABLE OF CONTENTS

INTRODUCTION

1.1 Background

The Endocrine Disruptor Screening Program (EDSP) reflects a two-tiered approach to implement the statutory testing requirements of FFDCA section 408(p) (21 U.S.C. 346a). 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 I screening assays will be used to identify substances that have the potential to interact with the estrogen, androgen, or thyroid hormone (Test guidelines in the OPPTS 890 series). The determination of a chemicals ability to interact with hormone systems will be made on a weight-of-evidence basis, taking into account data from the Tier 1 assays and other scientifically relevant information available. If a substance interacts with a hormone system, it does not imply that when used it will cause adverse effects in humans or ecological systems. The Uterotrophic Assay (OPPTS 890.1600) is used to screen substances for estrogenicity and is one of four *in vivo* mammalian assays in the EDSP Tier 1 battery of assays.

1.2 Purpose

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

1.3 Regulatory Compliance

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

17-α ethinyl estradiol will not be analyzed as stated in 40 CFR 160.105(b) of the U.S. EPA GLP requirements, a positive response in the test system following 17α-ethinyl estradiol administration will be evident following statistical analysis of the tissue weights.

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

1.4 Sponsor

NIEHS
P.O. Box 12233
Research Triangle Park, NC27709
[redacted]

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

NTP Investigator
[redacted]
Telephone No.:
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1.5 Testing Facility
Integrated Laboratory Systems, Inc. (ILS)

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

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

1.6 Study Dates

Animal Arrival Date:	May 9, 2011
Experimental Start Date:	May 16, 2011
Experimental Termination Date:	May 20, 2011

TEST SUBSTANCE, REFERENCE SUBSTANCE, VEHICLE

2.1 Test Substance: 2-Hydroxy-4-Methozybenzophenone (Oxybenzone)

CAS No.	131-57-7
Source:	Ivy Fine Chemicals Corporation
Lot/Batch No.:	20080801
ILS Repository No.:	11-29
Formula:	$C_{14}H_{12}O_3$
Description:	Light yellow powder

Purity:	99.9%
Expiration Date:	01 August 2012
Dose Formulation:	Corn Oil
Storage	
Test Substance:	Room Temperature
Dose Formulation:	1-10°C protected from light
Stability	
Dose Formulation:	Stable in corn oil for 42 days

2.2 Test Substance: 2-Ethylhexyl p-methozycinnamate (Octylmethoxycinnamate)

CAS No.	5466-77-3
Source:	Acros Organics
Lot/Batch No.:	A0293319
ILS Repository No.:	11-32
Formula:	$C_{18}H_{26}O_3$
Description:	Clear colorless liquid
Purity:	99.8%
Expiration Date:	04 July 2011
Dose Formulation:	Corn Oil
Storage	
Test Substance:	Room Temperature
Dose Formulation:	1-10°C protected from light
Stability	
Dose Formulation:	Stable in corn oil for 42 days

2.3 Test Substance: Octyl Salicylate (Octylsalate)

CAS No.	118-60-5
Source:	Sigma-Aldrich
Lot/Batch No.:	44698PJ
ILS Repository No.:	11-30
Formula:	$C_{15}H_{22}O_3$
Description:	Colorless liquid
Purity:	99.6%
Dose Formulation:	Corn Oil
Storage	
Test Substance:	Room Temperature
Dose Formulation:	1-10°C protected from light
Stability	
Dose Formulation:	Stable in corn oil for 42 days

2.4 Test Substance: 2-Ethylhexyl 2-Cyano-3,3-Diphenylacrylate (Octocrylene)

CAS No.	6197-30-4
Source:	Sigma-Aldrich
Lot/Batch No.:	01697MJ
ILS Repository No.:	11-31
Formula:	$C_{24}H_{27}NO_2$
Description:	Yellow viscous liquid
Purity:	99.2%

Dose Formulation:	Corn Oil
Storage	
Test Substance:	Room Temperature
Dose Formulation:	1-10°C protected from light
Stability	
Dose Formulation:	Stable in corn oil for 42 days

2.5 Reference Substance: 17α-Ethinyl Estradiol

CAS No.	57-63-6
Source:	Sigma Aldrich (St. Louis, MO)
Lot/Batch No.:	090M1241V
ILS Repository No.:	11-40
Formula:	$C_{20}H_{24}O_2$
Description:	White powder
Purity:	≥98%
Expiration Date:	February 2012
Dose Formulation:	ILS will prepare 17α-ethinyl estradiol in corn oil once at a dose level of 0.1mg/mL and dispense into amber vials to be used daily during the study.
Storage:	
Reference Substance:	Room temperature and protected from light
Dose Formulation:	1-10°C protected from light
Stability:	
Dose Formulation:	17α-ethinyl estradiol in corn oil stored between 1-10°C was shown to be stable for 42 days (Messer, 2002).

2.6 Vehicle: Corn Oil

CAS No.:	8001-30-7
Source:	MP Biomedicals, LLC (Solon, OH)
Lot/Batch No.:	7862K
ILS Repository No.:	11-94
Formula:	$C_{27}H_{50}O_6$
Description:	Yellow oil
Storage:	Room Temperature

2.7 Archival Samples

A ~1 g sample of the neat test substance, ~1 mg sample of the reference substance, and 1mL 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 archival samples will be discarded.

2.8 Dose Formulation Analysis

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

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

Oxybenzone, Octylsalate and Octocrylene:
Battelle Memorial Institute
[redacted]
TOXBC Test Article Custodian
651 W. Fifth Avenue
Columbus, OH 43201-2693

EXPERIMENTAL DESIGN

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

3.1 Test System

Species:	Rat, *Rattusnorvegicus*
Strain:	Sprague-DawleyCrl:CD®(SD) IGS
Source:	Charles River Laboratories International, Inc. (Raleigh, NC)
Number/Sex:	80 ovariectomized females, 6 weeks of age at ovariectomy. Surgical manipulation performed by Charles River Laboratories International, Inc.
Acclimation:	Animals will be allowed to recover from the surgical manipulation for 7 days at Charles River Laboratories International, Inc prior to shipment to ILS. The animals will then be acclimated to ILS for at least 7 days in the room where the study will occur.
Estrous Cycle:	Vaginal smears will be collected for 5 consecutive days immediately preceding dose administration and evaluated for stage of estrous cycle. If an animal indicates evidence of entering estrus, the animal will not be used on study.
Age at dose administration:	8-10 weeks of age
Weight at dose administration:	175-275 grams
Identification:	Each animal will be uniquely identified by ear punch prior to dose administration. Until the animals are ear punched, they will be identified by the temporary numbers located on the animal's cage.
Justification:	Animal model used is in accordance with OPPTS 890.1600: Uterotrophic Assay (U.S. EPA).

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, 1996).

Housing (pre-allocation):	1 per cage
Housing (post-allocation):	2 per cage
Cage Type:	Polycarbonate
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:	Once per week while single housed. Twice per week while multi-housed.
Diet:	Teklad Global 16% Protein Rodent Diet (Teklad Diets, Madison WI) *ad libitum* Prior to shipment, rats are given Autoclaved Purina5L79 Rat and Mouse diet *ad libitum* at Charles River Laboratories International, Inc. A copy of the diet composition will be included in the raw data.
Analysis:	The manufacturer's analytical results will be included in the raw data and reviewed prior to animal arrival to ensure the genistein equivalent content of genistein plus daidzein does not exceed 350 µg/g (Owens et al., 2003).
Archival:	A sample of the diet (~200 g) will be retained and stored between 0 and -30°C until acceptance of the final report and will then be discarded.
Water:	Reverse osmosis treated tap water (City of Durham, NC) *ad libitum*
Supplied:	Glass water bottles with stainless steel sipper 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: Once per week

Animal Room Conditions:

Temperature: 19-25°C

Humidity 30-70%

Lighting: 12/12 hour light/dark cycle

Animal Enrichment: None

3.3 Allocation

The animals will be assigned to a dose group using a procedure that stratifies animals across groups by body weight such that mean body weight of each group is not statistically different from any other group using analysis of variance (ANOVA) (Statistical Analysis System version 9.1,SAS Institute, Cary, NC). Only clinically healthy animals will be used for allocation.

3.4 Group Designations

Group Number	Animal Identification	Dose Group	Dose Level (mg/kg/day)
1	01-08	Corn Oil Control	0
2	09-16	Oxybenzone	320
3	17-24	Oxybenzone	1000
4	25-32	Octylmethoxycinnamate	320
5	33-40	Octylmethoxycinnamate	1000
6	41-48	Octylsalate	320
7	49-56	Octylsalate	1000
8	57-64	Octocrylene	320

Group Number	Animal Identification	Dose Group	Dose Level (mg/kg/day)
9	65-72	Octocrylene	1000
10	73-80	17α-Ethinyl Estradiol	0.1

3.5 Dose Administration

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

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

3.5.1 Justification of Route of Administration

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

3.5.2 Justification of Dose Levels

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

3.5.3 Disposal of Dose Formulations

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

3.6 In-Life Animal Observations

Mortality/Moribundity: Twice daily on weekdays, once daily on weekends/holidays

Clinical Observations: Observed within 2 days of arrival, again for allocation of animals to study groups, daily prior to dose administration, and prior to euthanasia.

A cage-side observation will occur 3 hours (± 30 minutes) after dose administration.

	If adverse clinical signs are seen additional observations may be recorded.
Body Weights:	Collected within 2 days of arrival, again for allocation of animals to study groups, daily prior to dose administration, and prior to euthanasia.

3.7 Termination

Moribunds/Unscheduled:	Tissue collection will not be performed on accidental deaths, moribund, or animals found dead during the acclimation period. Beginning on the first day of dose administration, any animals found moribund or dead will be necropsied under the supervision of a pathologist and cause of death will be determined and recorded, if possible. Moribund animals will be euthanized by carbon dioxide (CO_2) inhalation and death confirmed by cervical dislocation.
Scheduled:	Twenty four hours (± 2 hours) after the final dose administration, animals will be humanely euthanized, in the same order as they were dosed, by CO_2 asphyxiation with death confirmed by cervical dislocation.
Tissue Collection:	The uterus will be removed and the ends of the uterine horns will be examined for the presence of any ovarian tissue. If ovarian tissue is observed it will be noted in the study records. Gross observations of the uterus will be recorded.
Tissue Weights:	The uterus will be excised, trimmed of excess adhering tissue and fat, weighed, and weights recorded to the nearest 0.0001 g. The uterus will then be pierced and blotted to remove the luminal contents, weighed (blotted), and weights recorded to the nearest 0.0001 g.

3.8 Statistical Analysis

Descriptive statistics (mean and standard deviation) will be calculated using MS Excel. Final body weight, body weight gain, and tissue weights will be analyzed using Statistical Analysis System version 9.1 (SAS Institute, 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 uterine weights (wet and blotted weights) will be analyzed by ANOVA followed by pairwise comparisons using a Dunnett's one tailed t test. Statistically significant effects will be reported when $p < 0.05$.

3.9 Performance Criteria

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

REPORT

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

RECORD RETENTION

All original data [including the original signed study protocol and all amendments (if any), test substance information, animal receipt records, animal caretaker records, observations, body weight records, clinical observations, etc.], test substance archival sample, and the original final report will be maintained by ILS for 5 years following finalization of the study report. At the end of the 5 year period, sponsor will be contacted for the disposition of the study related materials.

REFERENCES

Institute of Laboratory Animal Resources. (1996). *Guide for the Care and Use of Laboratory Animals.*National Academy Press, Washington, DC.

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

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

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

KEY PERSONNEL

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

Appendix VIII:

Amendments, Deviations, and Notes to File

Integrated Laboratory Systems, Inc.

Protocol Amendment

ILS Project No.-Study No.: N135-231

Protocol Amendment No.: 1

Section Amended: ~~13~~ 2.1 (a)

Amendment Made: 2-Hydroxy-4-Methozybenzophenone (Oxybenzone) formulations will be stored at room temperature.

Reason for Amendment: The change is a result of the formulations at 200 mg/ml solidifying at refrigerated conditions. Formulations have been shown to be stable at room temperature.

[redacted] 5/13/11
Date
Chief Toxicology Branch
National Toxicology Program, NIEHS

[redacted] 5/13/11
Date
Contract Office Technical Representative
National Toxicology Program, NIEHS

[redacted] 5/10/11
Date
Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

[redacted] 5/16/11
Date
Principal ~~Study~~ Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

Page 1 of 1

(a)(1) [redacted] 5/10/11
(b) Corrected title [redacted] 8/15/11

Integrated Laboratory Systems, Inc.

Protocol Amendment

ILS Project No.-Study No.: N135-231

Protocol Amendment No.: 2

Section Amended: 1.3

Amendment Made: 17-α ethinyl estradiol will not be analyzed as stated in 40 CFR 160.113 (a) (1).

Reason for Amendment: Incorrect section of 40 CFR 160 was listed.

[redacted]
Chief Toxicology Branch
National Toxicology Program, NIEHS

6/10/11
Date

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

6/14/11
Date

[redacted]
Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

6/10/11
Date

[redacted]
Principal ~~Study~~ Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

6-20-11
Date

(a) corrected title
[redacted] 8/15/11

Integrated Laboratory Systems, Inc.

Protocol Amendment

ILS Project No.-Study No.: N135-231

Protocol Amendment No.: 3

Section Amended: 1.5

Amendment Made: Shipping address: 601 Keystone Park Drive, Suite 100
Durham, NC 27713

Reason for Amendment: Incorrect shipping address for testing facility was listed.

[redacted]

8/11/11
Date

Chief Toxicology Branch
National Toxicology Program, NIEHS

[redacted]

8/11/11
Date

Contract Office Technical Representative
National Toxicology Program, NIEHS

[redacted]

8/11/11
Date

Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

[redacted]

8/12/11
Date

Principal Study Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

(a) Correct title
[redacted] 8/15/11

Page 1 of 1

Integrated Laboratory Systems, Inc.

Protocol Amendment

ILS Project No.-Study No.: N135-231

Protocol Amendment No.: 4

Section Amended: Protocol

Amendment Made: The study will be reopened to include a protocol amendment.

Reason for Amendment: Change in disposition of original data by Sponsor.

Section Amended: Record Retention

Amendment Made: 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

Reason for Amendment: Change in disposition of original data by Sponsor.

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

4/18/12
Date

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

4/18/12
Date

[redacted]

Study Director
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

4-20-12
Date

[redacted]

Principal Toxicologist
Investigative Toxicology Division
Integrated Laboratory Systems, Inc.

4-20-12
Date

Integrated Laboratory Systems, Inc.

Protocol Deviation

ILS Project No.-Study No.: N135-231

Protocol Deviation No.: 1

Section Deviated:	2.7
Nature of Deviation:	No archival dose formulation sample was saved for the reference substance, 17α-ethinyl estradiol.
Reason for Deviation:	Inadvertently, no archival sample was removed from the formulation at the time the formulation was prepared.
Impact on Study:	None. Responses produced by the reference substance, 17α-ethinyl estradiol, in this study were within the acceptable range for this assay.

[redacted]

Study Director, ILS, Inc.

6-2011

Date

Integrated Laboratory Systems, Inc.

Protocol Deviation

ILS Project No.-Study No.: N135-231

Protocol Deviation No.: 2

Section Deviated: 3.1

Nature of Deviation: Body weights of animals below were greater than 275 grams at dose administration:

Animal Number	Body Weight
16	280.7
32	276.9
40	282.8
48	277.2
80	276.9

Reason for Deviation: The animals received from supplier were larger than anticipated.

Corrective Action: None.

Impact on Study: There should be no impact on study since there were no significant differences across dose groups when allocating animals.

[redacted]

Study Director, ILS, Inc.

8-10-11

Date

Integrated Laboratory Systems, Inc.

SOP Deviation

ILS Project No.-Study No.: N135-231

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

SOP Section Deviated:	II-A
Nature of Deviation:	Afternoon room check was not performed on 18 May 2011.
Reason for Deviation:	Oversight by technician.
Corrective Action:	Technician was reminded that the SOP states that room checks are to be performed twice daily on weekdays (at least 6 hours apart).
Impact on Study:	Clinical observations and daily mortality/moribundity checks did not show any adverse effects related to the missed room check.

[redacted]

Study Director, ILS, Inc.

6-14-11

Date

Integrated Laboratory Systems, Inc.

SOP Deviation

ILS Project No.-Study No.: N135-231

SOP No.-Mod. No. Deviated: 793-0

SOP Section Deviated:	II, A, 6
Nature of Deviation:	Prosectors did not sign and date the necropsy form for animal 02 on 19 May 2011 and animals 06 and 72 on 20 May 2011. Animal 72 also was not noted as having normal tissue.
Reason for Deviation:	Prosector oversight.
Corrective Action:	Prosectors were reminded to completely fill out necropsy forms.
Impact on Study:	There is no scientific impact on the study since the same prosectors were responsible for the same tissues for the entire necropsy. Although animal 72 was not specifically noted as being normal, no abnormal observations were made, which suggests the tissue was all normal. These tissue weights were also within 2 standard deviations of the mean tissue weight.

[redacted]

Study Director, ILS, Inc.

6-14-11

Date

Integrated Laboratory Systems, Inc.

Note to File

ILS Project No.-Study No.: N135-231

Note to File: 1

Lot/Batch number 20100801 from Ivy Fine Chemicals was used for dose formulations for Oxybenzone. The COA provided was from Lot/Batch number 20080801 which was included in the final signed protocol.

[redacted]

5-16-11
Date

Study Director, ILS, Inc.

Integrated Laboratory Systems, Inc.

Note to File

ILS Project No.-Study No.: N135-231

Note to File: 2

Group 10 (17α-ethinyl estradiol, positive control) was denoted as group 12 in the statistical analyses. All numbers in reference to group 12 are those of group 10.

[redacted]

6-2017
Date

Study Director, ILS, Inc.

Integrated Laboratory Systems, Inc.

Note to File

ILS Project No.-Study No.: N135-231

Note to File: 3

Dose formulations withdrawal log was misplaced for the 17α-ethinyl estradiol dose formulation. The formulations were removed and administered to the animals on study days 1-4, as verified by the daily dosing forms.

[redacted]

6·22·11
Date

Study Director, ILS, Inc.

Integrated Laboratory Systems, Inc.

Note to File

ILS Project No.-Study No.: N135-231

Note to File: 4

Dose formulations withdrawal log was misplaced for the 17α-ethinyl estradiol dose formulation. The formulations were removed and administered to the animals on study days 1-4, as verified by the daily dosing forms.

[redacted]

8-3-11
Date

Study Director, ILS, Inc.

Appendix IX:

Positive Control Test Data

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

Integrated Laboratory Systems, Inc.

Final Report Amendment

ILS Project No.-Study No.: N135-231

Final Report Amendment No.: 1

Amendment Made:	The final report is amended to include Protocol Amendment 4 as page 177 A and B.
Reason for Amendment:	Change in disposition of original data.
Amendment Made:	The final report is amended to include the page number on the first page.
Reason for Amendment:	Inadvertently omitted.
Amendment Made:	The Final Report Amendment was added to the Table of Contents.
Reason for Amendment:	Added to include in revised report.

[redacted]

Study Director 4-20-12 Date

[redacted]

Quality Assurance Officer 04/20/2012 Date

Page 1 of 1