CeeTox
In vitro models to predict toxicity

H295R Steroidogenesis Assay

Final Report

DATA REQUIREMENT(S): OPPTS 890.1550 (2009)

AUTHOR(S): [redacted]

STUDY COMPLETION DATE: 31 July 2013

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LABORATORY PROJECT ID:
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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study Number: 9070-100794STER

Study Title: H295R Steroidogenesis Assay

I, the undersigned, hereby declare that this study was performed in accordance with U.S. Environmental Protection Agency Good Laboratory Practice regulations Title 40 CFR 160 with the exception of section 160.113. Dose concentration of test and control substances were not verified using analytical methods.

The study was conducted according to the procedures herein described and this report represents a true and accurate record of the results obtained. There were no deviations that impacted the quality or integrity of the study data. Any deviations that occurred during the course of the study were noted in this report, with the full write-ups included in the study binder.

[redacted]

Study Director

31 July 2013

Date

FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Title: H295R Steroidogenesis Assay

Study Number: 9070-100794STER

In accordance with CeeTox, Inc.'s policies and Quality Assurance standard operating procedures for Good Laboratory Practice (GLP), the conduct of this study has been audited as follows:

Date(s) of Inspection/Audit	Inspection/Audit	Date(s) reported to Study Director	Date(s) reported to Management
12 Dec 2012	Draft protocol	12 Dec 2012	12 Dec 2012
05 Mar 2013	Draft protocol	05 Mar 2013	05 Mar 2013
09 Apr 2013	In process (compound preparation and dosing)	10 Apr 2013	10 Apr 2013
12 Apr 2013	In process (harvest and MTT)	17 Apr 2013	17 Apr 2013
08 Jul 2013 & 09 Jul 2013	Data Binder	09 Jul 2013	09 Jul 2013
09 Jul 2013 & 12 Jul 2013	Draft Report	15 Jul 2013	15 Jul 2013

The signature below indicates the summary table is an accurate representation of Quality Assurance's involvement with this study.

[redacted] 31 Jul 2013

Date

Quality Assurance Auditor
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GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
[redacted]	Study Director
	Director of Project Management
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	Laboratory Manager
	Cell Culture Scientist II
	Research Associate
	Associate Scientist

Study Dates

Study initiation date: 28 March 2013
Experimental start date: 02 April 2013
Experimental termination date: 25 April 2013

Deviations from the Protocol

See Appendix 12. There was one protocol deviation; however, the deviation did not impact the integrity of the data in this report.

Other

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

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

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1.0 EXECUTIVE SUMMARY

1.1 Study Design

The objective of this study was to evaluate the ability of four test substances to affect the steroidogenic pathway beginning with the sequence of reactions occurring after the gonadotropin hormone receptors through the production of testosterone and estradiol/estrone. The assay used the H295R human adrenocortical carcinoma cell line.

The four chemicals tested in the assay were 2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O), 2-Phenyl-5-benzimidazolesulfonic acid (Ensulizole), 3,3,5-Trimethylcyclohexyl Salicylate (Homosalate), Butyl-methoxydibenzoylmethane (Avobenzone). The final concentrations of each compound tested in the steroidogenesis assay were: 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 µM for Padimate O, Homosalate, and Avobenzone, and 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, and 10 µM for Ensulizole.

Five independent runs of the steroidogenesis assay were completed. Three of the five assays were analyzed for each compound. All test chemicals, reference chemicals, and solvent controls were tested in replicates of 6/plate. The H295R supplemented medium used in the assay at the time of plating, dosing, and harvest contained 10 µM 22R-hydroxycholesterol. The duration of exposure to each test chemical, reference chemical, or solvent control was 48 hours. A 48-well QC plate containing two doses of reference chemicals forskolin and prochloraz was run each time the assay was performed. Cell viability was assessed after the 48 hour exposure using the MTT assay. Testosterone and estradiol levels were measured using HPLC/MS-MS by OpAns, LLC (Durham, NC). All concentrations that exhibited greater than 20% cytotoxicity in the MTT cell viability assay were excluded from the statistical analysis of testosterone and estradiol levels.

1.2 Results

The three analyzed runs of the assay were conducted on April 2, 2013, April 10, 2013, and April 16, 2013. The results discussed in this report are from these three runs only.

The suitable highest concentration of Padimate O that could be analyzed in the assay was 10 µM in Runs 1 and 2 and 1 µM in Run 3 based on solubility results. Precipitate was noted at the 100 µM exposure concentration in Run 2 and at the 10 and 100 µM exposure concentrations in Run 3. Precipitation was not noted in Run 1. However, the 100 µM exposure concentration was excluded from the statistical analysis in this run as well based on the solubility results from the other two independent runs. In two of the three independent runs of the assay, no statistically significant changes in testosterone or estradiol were identified after Padimate O exposure at any of the concentrations that were analyzed. Statistically significant decreases in testosterone and estradiol were identified at the 0.1 µM Padimate O exposure concentration in Run 2. The

decreases were not dose dependent and were observed at one concentration only in this run. Statistically significant changes in testosterone or estradiol were not observed at any of the other exposure concentrations analyzed in Run 2 or in the other two runs of the assay.

The suitable highest concentration of Ensulizole that could be analyzed in the assay was 1 µM in Run 1 and 10 µM in Runs 2 and 3. In Run 1, precipitation was observed at the 10 µM exposure concentration. This concentration was excluded from the statistical analysis in this run. Cytotoxicity greater than 20% was not observed at any of the concentrations analyzed in the assay. In one independent run of the assay (Run 3), no statistically significant changes in testosterone or estradiol were observed. Statistically significant decreases in testosterone and estradiol were observed at the 0.1 µM Ensulizole exposure concentration in Run 1. In Run 2, statistically significant decreases in testosterone were observed at two exposure concentrations (0.001 and 0.01 µM) and statistically significant decreases in estradiol were observed at three exposure concentrations (0.001, 0.01, and 0.1 µM).

Homosalate was analyzed at exposure concentrations up to 10 µM. The 100 µM exposure concentration was excluded from the statistical analysis based on solubility results. Cytotoxicity greater than 20% was not observed at any of the concentrations analyzed in the assay. In two of the three independent runs of the assay, no statistically significant changes in testosterone or estradiol were observed at any of the Homosalate exposure concentrations that were analyzed. In the remaining run, statistically significant decreases in testosterone and estradiol were observed at one Homosalate exposure concentration (0.01 µM). Statistically significant changes were not observed at any of the other exposure concentrations in this run.

Avobenzone was evaluated at exposure concentrations up to 10 µM. The 100 µM exposure concentration was excluded from the analysis based on solubility and cytotoxicity results. Precipitation was noted in all three runs of the assay at the 100 µM exposure concentration. Cytotoxicity greater than 20% was also observed at this concentration in all three runs. In Run 1, no statistically significant changes in testosterone or estradiol were observed. In Run 2, statistically significant decreases in testosterone and estradiol were observed at multiple Avobenzone exposure concentrations (0.01, 0.1, 1, and 10 µM for testosterone; 0.1 and 1 µM for estradiol). In Run 3, a statistically significant decrease in testosterone was observed at the 1 µM Avobenzone exposure concentration, while significant decreases in estradiol were observed at the 0.1, 1, and 10 µM exposure concentrations.

1.3 Conclusions

According to the decision criteria and data interpretation procedure outlined in OECD Test Guideline 456, Padimate O is negative for effects on testosterone and estradiol at the concentrations evaluated in this H295R cell assay. No statistically significant effects were observed in two of the three independent runs of the assay, and the third run showed statistically significant effects on testosterone and estradiol at 0.1 µM only (equivocal).

A mean of the three independent runs was calculated for Ensulizole since statistically significant results were obtained but were not consistent across the three independent runs. When the average results for the effects of Ensulizole exposure on testosterone and estradiol were calculated, decreases (approximately 30%) was observed in both testosterone and estradiol at multiple Ensulizole exposure concentrations. The decreases in testosterone were not statistically significant at any of the concentrations analyzed. Statistically significant decreases in estradiol were observed at two non-adjacent Ensulizole exposure concentrations (0.01 and 1 μM).

According to the OECD decision criteria and data interpretation procedure, Homosalate is negative for effects on testosterone and estradiol at the concentrations evaluated in this H295R cell assay. No statistically significant effects were observed in two of the three independent runs of the assay, and the third run showed statistically significant effects on testosterone and estradiol at 0.01 μM only (equivocal).

In three independent runs, a negative run, an equivocal run, and a positive run were obtained for Avobenzone effects on testosterone. As a result, a mean of the three runs was calculated. After calculation of the mean fold change results, decreases (approximately 20-25%) in testosterone and estradiol were observed at multiple Avobenzone exposure concentrations. The decreases in testosterone were not statistically significant at any of the exposure concentrations analyzed. When the mean of the results of three independent runs was calculated for estradiol, the decreases in estradiol that were observed at multiple Avobenzone exposure concentrations were not statistically significant.

2.0 INTRODUCTION

2.1 Purpose

The objective of this study was to evaluate the ability of four test substances to affect the steroidogenic pathway beginning with the sequence of reactions occurring after the gonadotropin hormone receptors through the production of testosterone and estradiol/estrone using the H295R cell line.

The human H295R cell line is a human adrenocortical carcinoma cell line that expresses genes that encode for all the key enzymes for steroidogenesis.

2.2 Regulatory Citations

OPPTS 890.1550: Steroidogenesis (Human Cell Line – H295R). 2009.

3.0 MATERIALS AND METHODS

3.1 Test Substance

3.1.1 Test Substance Details

Test substance name:	2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O)
Test substance manufacturer:	Sigma-Aldrich
CAS number:	21245-02-3
Description:	Colorless liquid
Solvent used:	DMSO
Batch identification:	MKBF0590V
Expiry date:	Not provided
Purity:	98.1%
Molecular formula:	$C_{17}H_{27}NO_2$
Molecular weight:	277.4
Storage conditions:	Room Temperature

Test substance name:	2-Phenyl-5-benzimidazolesulfonic acid (Ensulizole)
Test substance manufacturer:	Sigma-Aldrich
CAS number:	27503-81-7
Description:	White powder
Solvent used:	DMSO
Batch identification:	05117JE
Expiry date:	Not provided
Purity:	99.6%
Molecular formula:	$C_{13}H_{10}N_2O_3S$
Molecular weight:	274.3
Storage conditions:	Room Temperature

Test substance name:	3,3,5-Trimethylcyclohexyl Salicylate (Homosalate)
Test substance manufacturer:	Spectrum Chemical Mfg. Corp
CAS number:	118-56-9
Description:	Colorless to light yellow liquid
Solvent used:	DMSO
Batch identification:	YT0976
Expiry date:	Not provided
Purity:	99.3%
Molecular formula:	$C_{16}H_{22}O_3$
Molecular weight:	262.34
Storage conditions:	Room Temperature

Test substance name:	Butyl-methoxydibenzoylmethane (Avobenzone)
Test substance manufacturer:	Universal Preserv-A-Chem Inc.
CAS number:	70356-09-1
Description:	Off White to Yellowish Crystalline Powder
Solvent used:	DMSO
Batch identification:	L802809
Expiry date:	Not provided
Purity:	98.5%
Molecular formula:	$C_{20}H_{22}O_3$
Molecular weight:	310.39
Storage conditions:	Room Temperature

Certificates of analysis for the test substances are presented in Appendix 13.

3.1.2 Vehicle Selection

Dimethyl sulfoxide (DMSO; Sigma Aldrich, lot numbers RNBC4600, RNBC5920) was selected as a suitable vehicle for all test substances. Forskolin and prochloraz were prepared on April 2, 2013, April 10, 2013, and April 16, 2013 for use in this study. Test chemicals were prepared in DMSO on April 2, 2013, April 10, 2013, and April 16, 2013 for use in this study. The 22R-hydroxycholesterol was prepared in ethanol on April 1, 2013, April 9, 2013, and April 15, 2013.

3.2 Control Substances

3.2.1 Forskolin

Source:	Sigma Aldrich
CAS number:	66575-29-9
Description:	White powder
Solvent used:	DMSO
Lot number:	SLBB5661V
Expiration date:	February 2018
Purity:	98%
Molecular formula:	$C_{22}H_{34}O_7$
Molecular weight:	410.50
Storage conditions:	Room Temperature

3.2.2 Prochloraz

Source:	Sigma Aldrich
CAS number:	67747-09-5
Description:	White powder
Solvent used:	DMSO
Lot number:	SZBA112X
Expiration date:	22 Apr 2015
Purity:	99.1%
Molecular formula:	$C_{15}H_{16}C_{13}N_3O_2$
Molecular weight:	376.67
Storage conditions:	Room Temperature

Certificates of analysis for the control substances are presented in Appendix 13.

3.2.3 Other Materials

3.2.3.1 22R-Hydroxycholesterol

Source:	Sigma Aldrich
CAS number:	17954-98-2
Description:	White powder
Solvent used:	Ethanol
Lot numbers:	081M4097V, 042M4067V
Retest Date:	July 2014, July 2014
Purity:	99.00%, 99.00%
Molecular weight:	402.65
Storage conditions:	Room Temperature

3.3 Cell Line

3.3.1 Source

The H295R cell line was used in this study. The cell line was obtained from the American Type Culture Collection (ATCC CLR-2128; Lot #7635054), Manassas, VA.

3.3.2 Stability of the Cell Line

The stability of the cell line was monitored by the use of the following reference chemicals: forskolin and prochloraz. Two concentrations for each reference chemical were included on a QC plate each time the assay was performed and the fold change values for testosterone and estradiol were compared to the acceptable values summarized below (values taken from the cited guideline). Additionally, basal production of testosterone and estradiol on the QC plate were compared to the acceptable values below (from EPA test guideline OPPTS 890.1550).

	Testosterone	**Estradiol**
Basal Production	≥5 times method detection limit	≥2.5 times method detection limit
Induction (10 µM Forskolin)	≥2 times solvent control	≥7.5 times solvent control
Inhibition (1 µM Prochloraz)	≤0.5 times solvent control	≤0.5 times solvent control

3.3.3 Cell Culture and Plating Conditions

Cells were maintained in Dulbecco's modified Eagle's medium/nutrient mixture F-12 Ham with 15 mM HEPES, sodium bicarbonate, ITS+Premix, and 2.5% Nu-Serum (Becton Dickinson, Catalog #355500, Lot #07483, 41217) in a 5% CO_2 incubator at approximately 37°C. H295R cells were grown for five passages, frozen in liquid nitrogen, then thawed and cultured for eight or nine additional passages prior to use in the assay. The culture medium was supplemented with 10 µM 22R-hydroxycholesterol at the time of plating, dosing, and harvest. The concentration of 22R-hydroxycholesterol was chosen based on laboratory proficiency experiments previously conducted at CeeTox. The cells were plated into wells of a 48-well cell culture plate at a density of 300,000 cells/mL (420 µL of cell suspension added to each well). The cells were then placed into a 5% CO_2 incubator at approximately 37°C for approximately 24 hours prior to chemical exposure.

3.4 Chemical Exposure and Assay Plate Organization

With the exception of ensulizole, the test substances were dissolved in DMSO to make 200 mM stocks and then serially diluted 1:10 in DMSO. Ensulizole was dissolve in DMSO to make a 20 mM stock and then serially diluted 1:10 in DMSO. 22R-hydroxycholesterol was dissolved in ethanol to make a 40 mM stock and then diluted in supplemented medium to a final concentration of 10 µM. The test substances were then diluted 1:2000 in supplemented medium containing 10 µM 22R-hydroxycholesterol to prepare mastermix solutions. Forskolin and prochloraz were dissolved in DMSO to make 20 mM solutions and then serially diluted in DMSO. Forskolin and prochloraz were then diluted 1:2000 in supplemented medium containing 10 µM 22R-hydroxycholesterol. When added to the cell culture plates, these dilutions yielded final concentrations of 1 µM and 10 µM for forskolin, 0.1 µM and 1 µM for prochloraz and 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 µM for Padimate O, Homosalate, and Avobenzone, and 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, and 10 µM for Ensulizole, with the final concentration of DMSO in the medium being held constant at 0.05% (v/v). The final ethanol concentration in the supplemented medium was 0.025% (v/v).

The cells were checked microscopically for adequate attachment and proper morphology prior to dosing. The medium was removed from the cells and replaced with 420 µL of medium containing 10 µM 22R-hydroxycholesterol and the concentrations of test substances indicated in the table below. All concentrations were tested in replicates of 6/plate. Assay plates were organized as detailed below:

For Padimate O, Homosalate, and Avobenzone:

	1	2	3	4	5	6	7	8
A	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM
B	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM
C	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM
D	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM
E	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM
F	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM

For Ensulizole:

	1	2	3	4	5	6	7	8
A	DMSO	Stock 1 10 µM	Stock 2 1 µM	Stock 3 0.1 µM	Stock 4 0.01 µM	Stock 5 0.001 µM	Stock 6 0.0001 µM	Stock 7 0.00001 µM
B	DMSO	Stock 1 10 µM	Stock 2 1 µM	Stock 3 0.1 µM	Stock 4 0.01 µM	Stock 5 0.001 µM	Stock 6 0.0001 µM	Stock 7 0.00001 µM
C	DMSO	Stock 1 10 µM	Stock 2 1 µM	Stock 3 0.1 µM	Stock 4 0.01 µM	Stock 5 0.001 µM	Stock 6 0.0001 µM	Stock 7 0.00001 µM
D	DMSO	Stock 1 10 µM	Stock 2 1 µM	Stock 3 0.1 µM	Stock 4 0.01 µM	Stock 5 0.001 µM	Stock 6 0.0001 µM	Stock 7 0.00001 µM
E	DMSO	Stock 1 10 µM	Stock 2 1 µM	Stock 3 0.1 µM	Stock 4 0.01 µM	Stock 5 0.001 µM	Stock 6 0.0001 µM	Stock 7 0.00001 µM
F	DMSO	Stock 1 10 µM	Stock 2 1 µM	Stock 3 0.1 µM	Stock 4 0.01 µM	Stock 5 0.001 µM	Stock 6 0.0001 µM	Stock 7 0.00001 µM

A concurrent quality control plate was included with each of the independent runs of the test substance plates. The QC plate was prepared and dosed in the same manner as the test chemicals with either forskolin or prochloraz according to the following plate map:

For QC Plates:

	1	2	3	4	5	6	7	8
A	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH
B	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH
C	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH
D	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH
E	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH
F	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH

After adding the reference substance/test substance dosing solutions, the plates were incubated in a 5% CO_2 incubator at approximately 37°C for approximately 48 hours. After the 48 hour exposure, each well was examined under the microscope and images were taken of the vehicle control wells as well as the two greatest non-cytotoxic concentrations (based on observation under the microscope). The media was collected from all wells in two equal aliquots and stored at approximately -80°C until shipment to the analytical laboratory for hormone measurement. After media removal, cell viability measured by MTT assay, described in section 3.5.1 below. In view of the short-term nature of studies of this type, no analyses of stability, homogeneity or achieved concentration(s) were carried out on preparations of the test substance or positive control chemicals, either before or after the treatment phase. This is not considered to have affected the integrity of the study. For the positive control compounds, stability is demonstrated by an appropriate response in the assay system.

3.5 Assays

3.5.1 Cytotoxicity Assay

Cell viability was monitored by MTT assay after the 48 hour exposure. On the QC plates, wells designated to receive methanol (control wells for cell death measurements) were rinsed twice with phosphate buffered saline (PBS), then incubated in methanol for 30 minutes at room temperature. After the methanol incubation, the methanol-treated wells were rinsed again with PBS three times. Following media removal and/or methanol treatment, 0.25 mL of 0.5 mg/mL MTT solution in supplemented medium containing 10 µM 22R-hydroxycholesterol was added to each well of the test chemical and QC plates. The plates were incubated at approximately 37°C in a 5% CO_2 incubator for 3 hours. Following the 3 hour incubation, the MTT solution was removed from each well and 0.25 mL of isopropanol was added to each well. Plates were incubated at room temperature for 20 minutes with shaking. Following this incubation, absorbance at 570 nm and 650 nm were measured on a BioTek Synergy plate reader. The

absorbance at 650 nm was subtracted from the absorbance at 570 nm to calculate the MTT value for each well.

The change in cell viability was determined by comparing treated wells to the solvent control wells. A greater than 20% reduction in cell viability was considered evidence of cytotoxicity.

3.5.2 Precipitation Assay

Final dilutions of test substance in supplemented media were observed visually for evidence of precipitation. If precipitation was observed, the concentration was considered insoluble and was excluded from further analysis. Additionally, test substance solubility was evaluated by nephelometry before and after the exposure period.

3.5.3 Hormone Measurement System

Testosterone and estradiol levels were measured using HPLC/MS-MS at OpAns, LLC (Durham, NC). The method detection limit is 100 pg/mL for testosterone and 10 pg/mL for estradiol. The resulting minimum basal production levels based on the specifications in the test guideline (OPPTS 890.1550) are 500 pg/mL for testosterone and 25 pg/mL for estradiol. The report from this test site is provided as Appendix 14 in this report.

3.6 Data Analysis and Interpretation

3.6.1 QC Plates

Mean values (pg/mL) and standard deviations for testosterone and estradiol were calculated for each concentration of the reference chemicals and the solvent controls, as well as for the blank and background wells. Relative changes in hormone production were calculated using the following equation:

Relative Change = [Hormone] in each well ÷ [Hormone] of mean solvent (vehicle) control

For forskolin induction of testosterone, the background hormone production was subtracted from the forskolin-treated wells and blank and solvent control wells before calculating the relative change. Background hormone production was calculated from three wells with cells on the QC plate that received no 22R-hydroxycholesterol at the time of exposure.

3.6.2 Test Chemical Plates

Mean values (pg/mL) and standard deviations for testosterone and estradiol were calculated for each concentration of the test chemical, reference chemicals, and the solvent controls. Relative changes in testosterone and estradiol production were calculated using the equation below:

Relative Change = [Hormone] in each well ÷ [Hormone] of mean solvent (vehicle) control

All concentrations that exhibited greater than 20% cytotoxicity in the MTT cell viability assay were excluded from further analysis. Concentrations where precipitation was observed were also excluded from further analysis.

Prior to conducting statistical analyses, Dixon Q test for outlier identification and rejection was performed on groups of replicates with coefficients of variation greater than 30%. Normality of the data was evaluated using Shapiro-Wilk's test. Homogeneity of the variances between the treatment groups was evaluated using Levene's test. If the p-values were greater than 0.05 in both tests, statistical significance between each treatment group and the control group was evaluated using Dunnett's test. If the p-values were less than or equal to 0.05 in either the normality or the homogeneity test, a log transformation was performed on the data to attempt to approximate a normal distribution. If, following the transformation, p-values were greater than 0.05 in both the normality and homogeneity tests, Dunnett's test was performed on the transformed data to evaluate statistical significance between each treatment group and the control group. If, following the log transformation, p-values were less than or equal to 0.05 in either the normality or the homogeneity test, the non-transformed data set was analyzed using the nonparametric Kruskal-Wallis test followed by Dunn's test to evaluate statistical significance between each treatment group and the control group.

4.0 RESULTS AND DISCUSSION

4.1 Concentration Range for the Test Substance

Padimate O, Homosalate, and Avobenzone were tested at the following concentrations: 0.0001, 0.001, 0.01, 0.1, 1, 10, and 100 µM. Ensulizole was tested at the following concentrations: 0.00001, 0.0001, 0.001, 0.01, 0.1, 1, and 10 µM.

4.2 Assay Acceptance Criteria

In all three independent runs of the assay, the basal production of testosterone and estradiol on the quality control plates were above the required levels specified in section 3.3.2. In addition, the fold change required after induction with 10 µM forskolin and inhibition with 1 µM prochloraz on the quality control plates met the requirements specified in section 3.3.2 for both the testosterone and estradiol analyses.

The coefficients of variation (CV) for solvent control replicate wells for testosterone and estradiol within a plate based on absolute concentrations were less than 30% as specified in the test guideline (OPPTS 890.1550) for all test substance plates with the exception of two test

substance plates. The CV for solvent control replicate wells for estradiol on the avobenzone plate in Run 1 was 32.7% and the CV for solvent control replicate wells for testosterone on the Padimate O plate in Run 3 was 31.6%.

The between plate coefficient of variation for solvent controls based on fold change was 20.14% for testosterone and 17.81% for estradiol for Padimate O. The between plate coefficient of variation for solvent controls for Homosalate based on fold change was 15.15% for testosterone and 14.18% for estradiol. For Avobenzone, the between plate coefficient of variation for solvent controls based on fold change was 19.23% for testosterone and 19.55% for estradiol. For Ensulizole, the between plate coefficient of variation for solvent controls based on fold change was 13.89% for testosterone and 15.61% for estradiol. The between plate coefficients of variation fall within the specifications outlined in the test guideline for all compounds tested.

4.3 Assay Results and Discussion

The suitable highest concentration of Padimate O that could be analyzed in the assay was 10 µM in Runs 1 and 2 and 1 µM in Run 3 based on solubility results. Precipitate was noted at the 100 µM exposure concentration in Run 2 and at the 10 and 100 µM exposure concentrations in Run 3. Precipitation was not noted in Run 1. However, the 100 µM exposure concentration was excluded from the statistical analysis in this run as well based on the solubility results from the other two independent runs. In two of the three independent runs of the assay, no statistically significant changes in testosterone or estradiol were identified after Padimate O exposure at any of the concentrations that were analyzed. Statistically significant decreases in testosterone and estradiol were identified at one Padimate O exposure concentration (0.1 µM) in Run 2. The decreases were not dose dependent and were observed at one concentration only in this run. Statistically significant changes in testosterone or estradiol were not observed at any of the other exposure concentrations analyzed in Run 2 or in the other two runs of the assay.

The suitable highest concentration of Ensulizole that could be analyzed in the assay was 1 µM in Run 1 and 10 µM in Runs 2 and 3. In Run 1, precipitation was observed at the 10 µM exposure concentration. This concentration was excluded from the statistical analysis in this run. Cytotoxicity greater than 20% was not observed at any of the concentrations analyzed in the assay. In one independent run of the assay (Run 3), no statistically significant changes in testosterone or estradiol were observed. Statistically significant decreases in testosterone and estradiol were observed at one Ensulizole exposure concentration in Run 1 (0.1 µM). In Run 2, statistically significant decreases in testosterone were observed at two exposure concentrations (0.001 and 0.01 µM) and statistically significant decreases in estradiol were observed at three exposure concentrations (0.001, 0.01, and 0.1 µM).

Homosalate was analyzed at exposure concentrations up to 10 µM. The 100 µM exposure concentration was excluded from the statistical analysis based on solubility results. Cytotoxicity greater than 20% was not observed at any of the concentrations analyzed in the assay. In two of

the three independent runs of the assay, no statistically significant changes in testosterone or estradiol were observed at any of the Homosalate exposure concentrations that were analyzed. In the remaining run, statistically significant decreases in testosterone and estradiol were observed at one Homosalate exposure concentration (0.01 μM). Statistically significant changes were not observed at any of the other exposure concentrations in this run.

Avobenzone was evaluated at exposure concentrations up to 10 μM. The 100 μM exposure concentration was excluded from the analysis based on solubility and cytotoxicity results. Precipitation was noted in all three runs of the assay at the 100 μM exposure concentration. Cytotoxicity greater than 20% was also observed at this concentration in all three runs. In Run 1, no statistically significant changes in testosterone or estradiol were observed. In Run 2, statistically significant decreases in testosterone and estradiol were observed at multiple Avobenzone exposure concentrations (0.01, 0.1, 1, and 10 μM for testosterone; 0.1 and 1 μM for estradiol). In Run 3, a statistically significant decrease in testosterone was observed at the 1 μM Avobenzone exposure concentration, while significant decreases in estradiol were observed at the 0.1, 1, and 10 μM exposure concentrations.

5.0 CONCLUSIONS

According to the decision criteria and data interpretation procedure outlined in OECD Test Guideline 456, Padimate O is negative for effects on testosterone and estradiol at the concentrations evaluated in this H295R cell assay. No statistically significant effects were observed in two of the three independent runs of the assay, and the third run showed statistically significant effects on testosterone and estradiol at 0.1 μM only (equivocal).

A mean of the three independent runs was calculated for Ensulizole since statistically significant results were obtained but were not consistent across the three independent runs. When the average results for the effects of Ensulizole exposure on testosterone and estradiol were calculated, decreases (approximately 30%) was observed in both testosterone and estradiol at multiple Ensulizole exposure concentrations. The decreases in testosterone were not statistically significant at any of the concentrations analyzed. Statistically significant decreases in estradiol were observed at two non-adjacent Ensulizole exposure concentrations (0.01 and 1 μM).

According to the OECD decision criteria and data interpretation procedure, Homosalate is negative for effects on testosterone and estradiol at the concentrations evaluated in this H295R cell assay. No statistically significant effects were observed in two of the three independent runs of the assay, and the third run showed statistically significant effects on testosterone and estradiol at 0.01 μM only (equivocal).

In three independent runs, a negative run, an equivocal run, and a positive run were obtained for Avobenzone effects on testosterone. As a result, a mean of the three runs was calculated. After calculation of the mean fold change results, decreases (approximately 20-25%) in testosterone

and estradiol were observed at multiple Avobenzone exposure concentrations. The decreases in testosterone were not statistically significant at any of the exposure concentrations analyzed. When the mean of the results of three independent runs was calculated for estradiol, the decreases in estradiol that were observed at multiple Avobenzone exposure concentrations were not statistically significant.

6.0 REFERENCES

Endocrine Disruptor Screening Program Test Guidelines. *OPPTS 890.1550: Steroidogenesis (Human Cell Line – H295R)*. EPA 640-C-09-003. October 2009.

OECD Guideline for the Testing of Chemicals. Test Guideline 456. H295R Steroidogenesis Assay. 28 July 2011.

TABLES SECTION

TABLE 1 Results of MTT Cell Viability Assay – QC Plate

Condition	Cell Viability – Run 1 (% of SC)		Cell Viability - Run 2 (% of SC)		Cell Viability – Run 3 (% of SC)	
	Mean	**SD**	**Mean**	**SD**	**Mean**	**SD**
Blank	95.4	10.99	88.8	14.08	97.7	1.80
Background	93.5	7.83	94.5	2.41	95.9	0.98
SC + Methanol	6.1	1.12	6.3	0.45	6.0	0.61
Forskolin 1 µM	105.4	1.72	102.7	3.25	102.9	1.31
Forskolin 10 µM	107.3	3.44	104.4	1.79	104.6	2.63
Prochloraz 0.1 µM	101.2	5.17	97.9	1.41	99.4	2.29
Prochloraz 1 µM	101.1	4.11	99.3	2.24	99.3	2.94

SC = Solvent Control
SD = Standard Deviation

TABLE 2 Results of MTT Cell Viability Assay – Padimate O

Concentration (µM)	Cell Viability – Run 1 (% of SC)		Cell Viability – Run 2 (% of SC)		Cell Viability – Run 3 (% of SC)	
	Mean	SD	Mean	SD	Mean	SD
0.0001	108.0	6.52	102.0	1.54	102.6	0.87
0.001	112.3	8.21	103.4	1.35	100.5	2.37
0.01	111.9	6.59	103.8	1.51	100.2	0.90
0.1	112.1	5.04	105.0	2.45	100.3	1.81
1	113.5	3.60	103.5	1.79	100.5	2.57
10	110.2	3.38	105.6	1.62	101.3	1.84
100	100.5	4.35	93.5	2.27	98.8	3.68

SD = Standard Deviation

TABLE 3 Results of MTT Cell Viability Assay - Ensulizole

Concentration (µM)	Cell Viability – Run 1 (% of SC)		Cell Viability – Run 2 (% of SC)		Cell Viability – Run 3 (% of SC)	
	Mean	SD	Mean	SD	Mean	SD
0.00001	103.1	6.54	102.4	4.47	98.4	0.99
0.0001	106.7	6.93	101.1	2.02	97.0	1.38
0.001	106.0	6.49	101.9	1.44	98.2	0.98
0.01	107.1	4.29	102.4	2.71	98.4	1.44
0.1	106.4	2.85	102.8	1.58	97.2	3.58
1	106.2	2.37	101.8	1.64	97.3	1.79
10	105.8	1.74	102.6	1.68	97.9	2.49

SD = Standard Deviation

TABLE 4 Results of MTT Cell Viability Assay - Homosalate

Concentration (µM)	Cell Viability – Run 1 (% of SC)		Cell Viability – Run 2 (% of SC)		Cell Viability – Run 3 (% of SC)	
	Mean	SD	Mean	SD	Mean	SD
0.0001	107.9	6.37	100.2	2.97	102.2	1.26
0.001	110.6	8.97	100.4	2.64	101.6	1.04
0.01	110.3	6.70	99.0	4.11	102.0	1.78
0.1	110.7	4.61	99.3	1.91	101.6	3.17
1	110.6	3.20	99.3	2.00	101.3	2.58
10	107.4	4.41	97.5	1.74	99.5	1.94
100	92.8	17.79	87.0	2.54	90.6	4.41

SD = Standard Deviation

TABLE 5 Results of MTT Cell Viability Assay - Avobenzone

Concentration (µM)	Cell Viability – Run 1 (% of SC)		Cell Viability – Run 2 (% of SC)		Cell Viability – Run 3 (% of SC)	
	Mean	SD	Mean	SD	Mean	SD
0.0001	109.6	6.57	99.6	1.70	102.9	0.80
0.001	110.7	9.57	102.1	2.49	102.4	0.92
0.01	110.9	6.28	101.5	1.67	101.3	2.76
0.1	111.8	5.10	101.6	2.31	101.0	1.71
1	109.5	3.53	99.7	2.04	98.7	1.83
10	98.0	2.19	86.7	2.04	87.4	0.77
100	62.2*	9.56	68.5*	2.82	74.3*	1.92

SD = Standard Deviation

* Concentration excluded from statistical analysis because of cytotoxicity greater than 20%.

TABLE 6 QC Plate Raw Data and Fold Change Results – Testosterone

	Average Testosterone (pg/mL)			Testosterone Fold Change over SC			Testosterone Fold Change over SC – Background Subtracted		
Concentration	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**
Background	1455	1347	1513	0.49	0.49	0.47	N/A*	N/A*	N/A*
Blank	2809	2678	3225	0.95	0.97	1.01	0.90	0.95	1.01
DMSO	2955	2753	3203	1.00	1.00	1.00	1.00	1.00	1.00
1 µM Forskolin	5834	4102	5139	1.97	1.49	1.60	2.92	1.96	2.15
10 µM Forskolin	7508	5572	6133	2.54	2.02	1.92	4.03	3.00	2.73
0.1 µM Prochloraz	2340	2055	2802	0.79	0.75	0.87	N/A*	N/A*	N/A*
1 µM Prochloraz	1008	929	1177	0.34	0.34	0.37	N/A*	N/A*	N/A*

For forskolin induction of testosterone, background hormone concentration is subtracted from all other concentrations prior to calculating fold change values.
*N/A = not applicable

TABLE 7 QC Plate Raw Data and Fold Change Results – Estradiol

	Average Estradiol (pg/mL)			Estradiol Fold Change over SC		
Concentration	**Run 1**	**Run 2**	**Run 3**	**Run 1**	**Run 2**	**Run 3**
Background	ND*	ND*	12*	ND*	ND*	ND*
Blank	71	54	60	1.04	0.97	1.08
DMSO	68	56	55	1.00	1.00	1.00
1 µM Forskolin	418	436	650	6.14	7.82	11.75
10 µM Forskolin	758	862	1176	11.15	15.45	21.27
0.1 µM Prochloraz	61	48	58	0.89	0.85	1.05
1 µM Prochloraz	26	21	28	0.38	0.38	0.51

*ND = values below the detection limit for estradiol. For Run 3, the background average is the average of 5 values as the sixth value was below the detection limit.

TABLE 8 Quality Control Plate Results for Testosterone

	Run 1	Run 2	Run 3
Basal Production – Blank Wells	2809 pg/mL	2678 pg/mL	3225 pg/mL
Basal Production – Solvent Control Wells	2955 pg/mL	2753 pg/mL	3203 pg/mL
Induction (10 µM Forskolin)	4-fold	3-fold	3-fold
Inhibition (1 µM Prochloraz)	0.3-fold	0.3-fold	0.4-fold

For forskolin induction of testosterone, background hormone concentration is subtracted from all other concentrations prior to calculating fold change values.

TABLE 9 Quality Control Plate Results for Estradiol

	Run 1	Run 2	Run 3
Basal Production – Blank Wells	71 pg/mL	54 pg/mL	60 pg/mL
Basal Production – Solvent Control Wells	68 pg/mL	56 pg/mL	55 pg/mL
Induction (10 µM Forskolin)	11.2-fold	15.5-fold	21.3-fold
Inhibition (1 µM Prochloraz)	0.4-fold	0.4-fold	0.5-fold

TABLE 10 Padimate O – Results for Testosterone

Concentration (µM)	Fold Change over SC Run 1		Fold Change over SC Run 2		Fold Change over SC Run 3	
	Mean	SD	Mean	SD	Mean	SD
0.0001	1.05	0.06	0.97	0.04	1.29	0.25
0.001	0.97	0.10	0.99	0.03	1.12	0.26
0.01	1.01	0.16	0.86	0.12	1.01	0.29
0.1	0.88	0.15	0.76*	0.12	0.85	0.21
1	0.97	0.13	0.80	0.22	0.91	0.17
10**	1.04	0.15	0.93	0.13	N/A**	N/A**

SC = Solvent Control

SD = Standard Deviation

*Denotes statistical significance ($p \leq 0.05$).

**10 µM was the highest concentration of Padimate O analyzed in Runs 1 and 2 because of precipitation at the 100 µM concentration. In Run 3, 1 µM was the highest concentration analyzed because precipitation was noted at 10 and 100 µM.

TABLE 11 Padimate O – Results for Estradiol

Concentration (µM)	Fold Change over SC Run 1		Fold Change over SC Run 2		Fold Change over SC Run 3	
	Mean	SD	Mean	SD	Mean	SD
0.0001	0.98	0.06	0.90	0.06	1.28	0.22
0.001	0.91	0.10	0.94	0.05	1.15	0.25
0.01	0.97	0.15	0.83	0.11	1.07	0.31
0.1	0.87	0.15	0.71*	0.13	0.90	0.16
1	0.96	0.12	0.76	0.17	1.02	0.19
10**	1.19	0.15	1.10	0.15	N/A**	N/A**

SC = Solvent Control
SD = Standard Deviation
*Denotes statistical significance ($p \leq 0.05$).
**10 µM was the highest concentration of Padimate O analyzed in Runs 1 and 2 because of precipitation at the 100 µM concentration. In Run 3, 1 µM was the highest concentration analyzed because precipitation was noted at 10 and 100 µM.

TABLE 12 Ensulizole – Results for Testosterone

Concentration (µM)	Fold Change over SC Run 1		Fold Change over SC Run 2		Fold Change over SC Run 3		Mean of 3 Runs	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0.00001	0.86	0.08	1.02	0.08	1.20	0.07	1.03	0.17
0.0001	0.85	0.20	0.88	0.22	1.03	0.27	0.92	0.09
0.001	0.91	0.12	0.53*	0.12	0.99	0.20	0.81	0.25
0.01	0.74	0.18	0.47*	0.04	0.83	0.15	0.68	0.19
0.1	0.73*	0.22	0.74	0.30	0.95	0.19	0.81	0.13
1	0.79	0.24	0.54	0.04	0.84	0.21	0.72	0.16
10**	N/A**	N/A**	0.72	0.22	0.93	0.23	0.82	0.15

SC = Solvent Control
SD = Standard Deviation
*Denotes statistical significance ($p \leq 0.05$).
**1 µM was the highest concentration of Ensulizole analyzed in Run1 because of precipitation at the 10 µM exposure concentration in this run.

TABLE 13 Ensulizole – Results for Estradiol

Concentration (µM)	Fold Change over SC Run 1		Fold Change over SC Run 2		Fold Change over SC Run 3		Mean of 3 Runs	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD
0.00001	0.88	0.11	0.96	0.05	1.08	0.06	0.97	0.10
0.0001	0.85	0.22	0.86	0.18	0.96	0.28	0.89	0.06
0.001	0.91	0.13	0.51*	0.11	0.92	0.21	0.78	0.24
0.01	0.78	0.19	0.47*	0.06	0.77	0.14	0.67*	0.18
0.1	0.72*	0.22	0.73	0.29	0.87	0.19	0.77	0.08
1	0.77	0.23	0.53*	0.03	0.80	0.21	0.70*	0.15
10**	N/A**	N/A**	0.82	0.34	0.91	0.27	0.87	0.07

SC = Solvent Control
SD = Standard Deviation
*Denotes statistical significance ($p \leq 0.05$).
**1 µM was the highest concentration of Ensulizole analyzed in Run1 because of precipitation at the 10 µM exposure concentration in this run.

TABLE 14 Homosalate – Results for Testosterone

Concentration (µM)	Fold Change over SC Run 1		Fold Change over SC Run 2		Fold Change over SC Run 3	
	Mean	SD	Mean	SD	Mean	SD
0.0001	0.96	0.10	1.00	0.11	1.24	0.20
0.001	0.87	0.17	0.98	0.14	1.13	0.22
0.01	0.89	0.19	0.77*	0.17	0.86	0.35
0.1	0.88	0.18	0.88	0.12	0.95	0.24
1	0.85	0.17	0.79	0.16	0.89	0.22
10**	0.87	0.17	0.83	0.10	0.81	0.11

SC = Solvent Control

SD = Standard Deviation

*Denotes statistical significance ($p \leq 0.05$).

**10 µM was the highest concentration of Homosalate analyzed in all three runs of the assay because of precipitation at the 100 µM exposure concentration.

TABLE 15 Homosalate – Results for Estradiol

Concentration (µM)	Fold Change over SC Run 1		Fold Change over SC Run 2		Fold Change over SC Run 3	
	Mean	SD	Mean	SD	Mean	SD
0.0001	0.84	0.11	0.94	0.14	1.13	0.16
0.001	0.78	0.16	0.93	0.15	1.04	0.19
0.01	0.80	0.16	0.74*	0.16	0.81	0.33
0.1	0.84	0.17	0.91	0.16	0.88	0.22
1	0.80	0.16	0.83	0.18	0.85	0.18
10**	0.94	0.18	1.02	0.13	0.92	0.09

SC = Solvent Control

SD = Standard Deviation

*Denotes statistical significance ($p \leq 0.05$).

**10 µM was the highest concentration of Homosalate analyzed in all three runs of the assay because of precipitation at the 100 µM exposure concentration.

TABLE 16 Avobenzone – Results for Testosterone

Concentration (µM)	Fold Change over SC Run 1		Fold Change over SC Run 2		Fold Change over SC Run 3		Mean of 3 Runs	
	Mean	SD	Mean	Mean	SD	SD	Mean	SD
0.0001	1.31	0.09	0.91	0.10	1.15	0.07	1.12	0.20
0.001***	1.28	0.10	0.91	0.10	0.95	0.25	1.05	0.20
0.01	1.08	0.26	0.73*	0.16	0.89	0.14	0.90	0.17
0.1	0.94	0.31	0.70*	0.18	0.79	0.13	0.81	0.12
1	1.00	0.29	0.64*	0.18	0.74*	0.18	0.79	0.19
10**	1.09	0.26	0.67*	0.19	0.79	0.14	0.85	0.21

SC = Solvent Control
SD = Standard Deviation
*Denotes statistical significance ($p \leq 0.05$).
**10 µM was the highest concentration of Avobenzone analyzed in all three runs of the assay because of precipitation and cytotoxicity greater than 20% at the 100 µM exposure concentration.
***One contaminated well was excluded from the analysis at this exposure concentration in Run 3.

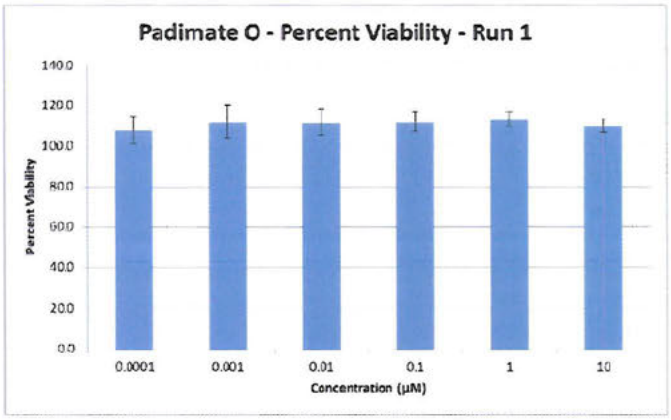
TABLE 17 Avobenzone – Results for Estradiol

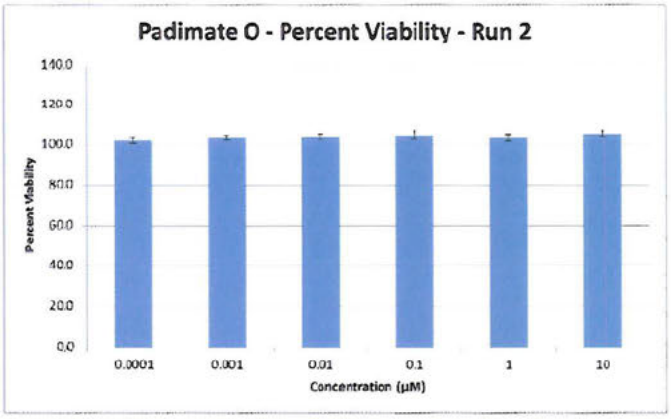
Concentration (µM)	Fold Change over SC Run 1		Fold Change over SC Run 2		Fold Change over SC Run 3		Mean of 3 Runs	
	Mean	SD	Mean	Mean	SD	SD	Mean	SD
0.0001	1.20	0.11	0.85	0.13	1.03	0.07	1.03	0.17
0.001***	1.25	0.15	0.88	0.11	0.86	0.24	1.00	0.22
0.01	1.06	0.29	0.69	0.13	0.80	0.14	0.85	0.19
0.1	0.89	0.25	0.65*	0.14	0.71*	0.13	0.75	0.12
1	0.96	0.26	0.52*	0.04	0.69*	0.16	0.72	0.22
10**	1.04	0.21	0.73	0.19	0.77*	0.14	0.85	0.17

SC = Solvent Control
SD = Standard Deviation
*Denotes statistical significance ($p \leq 0.05$).
**10 µM was the highest concentration of Avobenzone analyzed in all three runs of the assay because of precipitation and cytotoxicity greater than 20% at the 100 µM exposure concentration.
***One contaminated well was excluded from the analysis at this exposure concentration in Run 3.

FIGURES SECTION

FIGURE 1 Padimate O – MTT Cell Viability Results





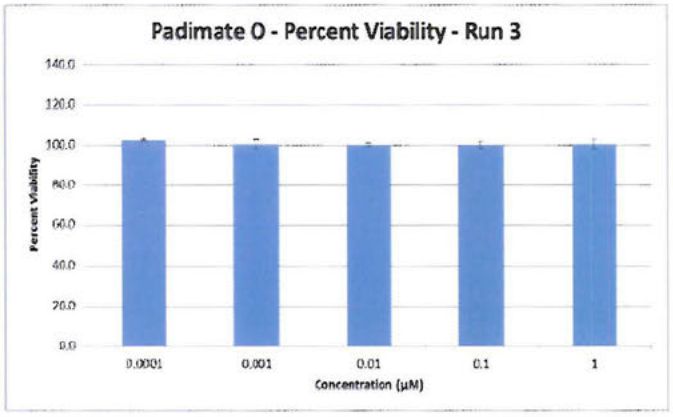
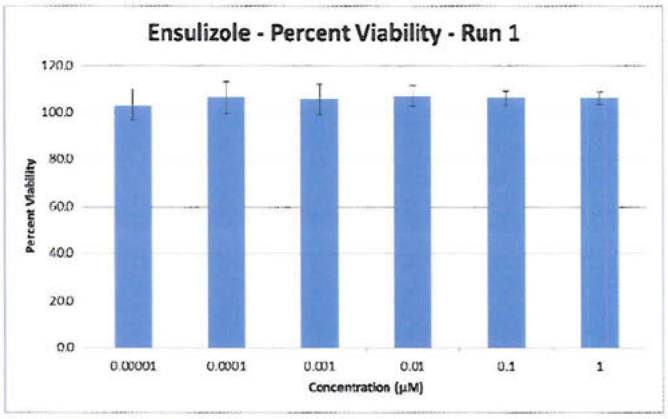
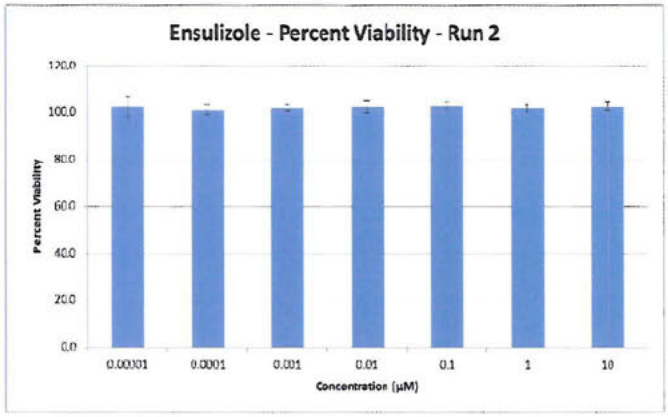


FIGURE 2 Ensulizole – MTT Cell Viability Results





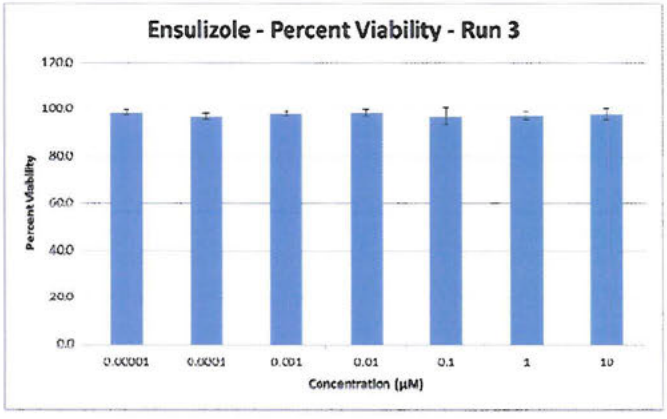
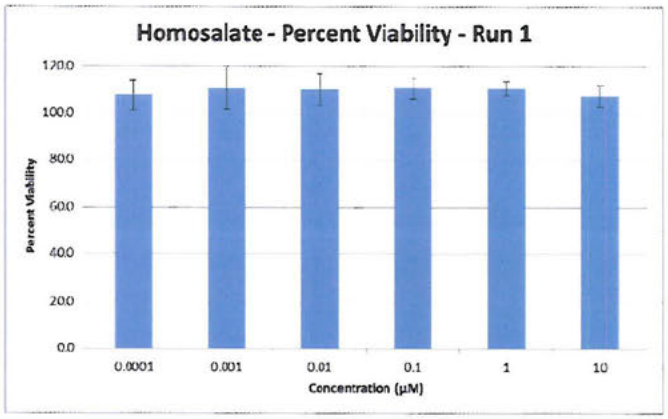
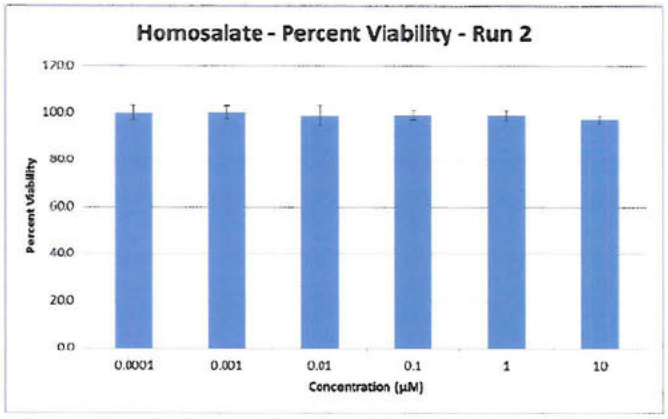


FIGURE 3 Homosalate – MTT Cell Viability Results





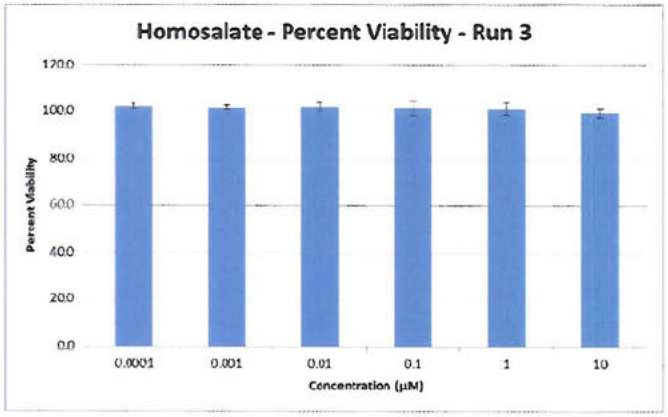
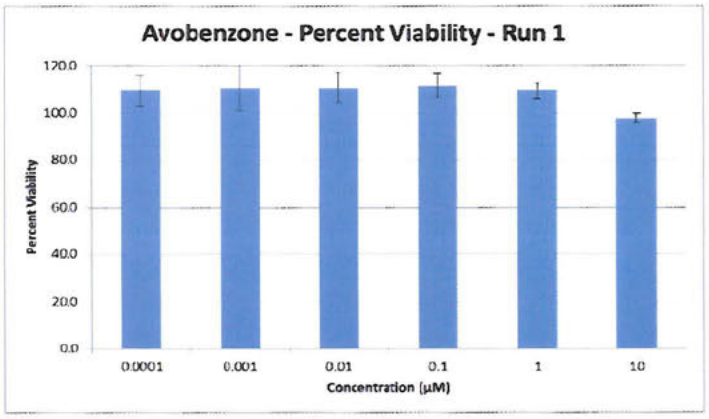
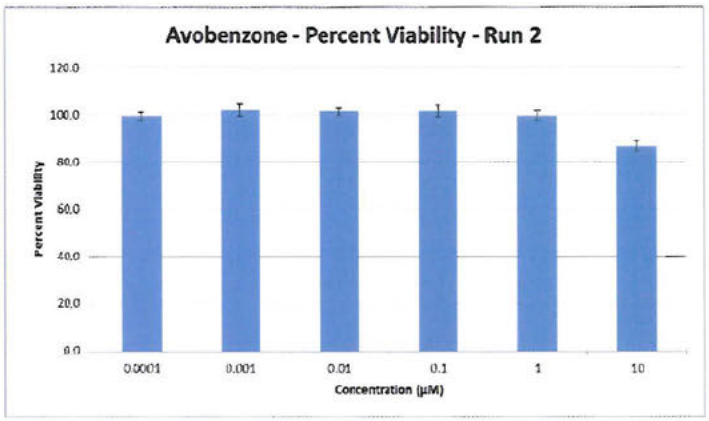


FIGURE 4 Avobenzone – MTT Cell Viability Results





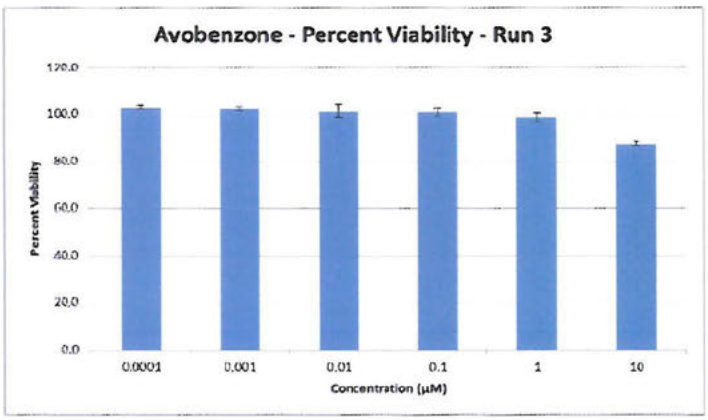
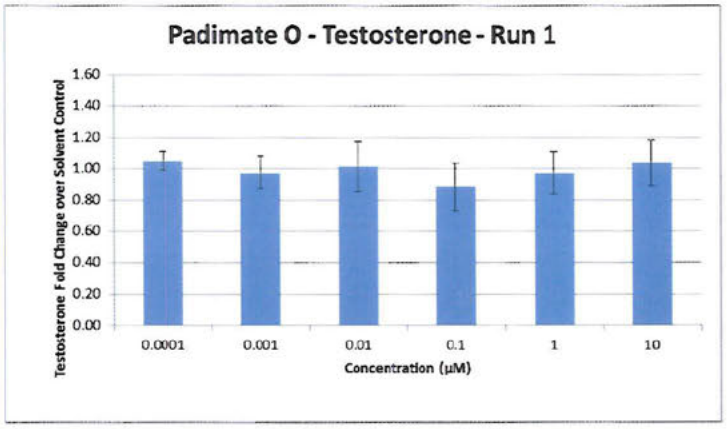
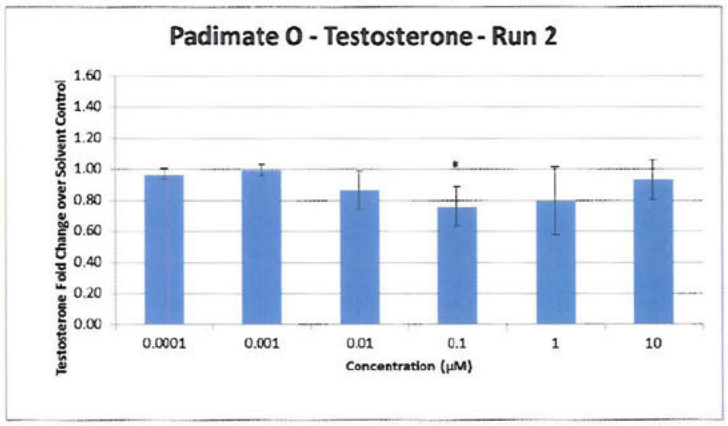


FIGURE 5 Padimate O – Testosterone Fold Change





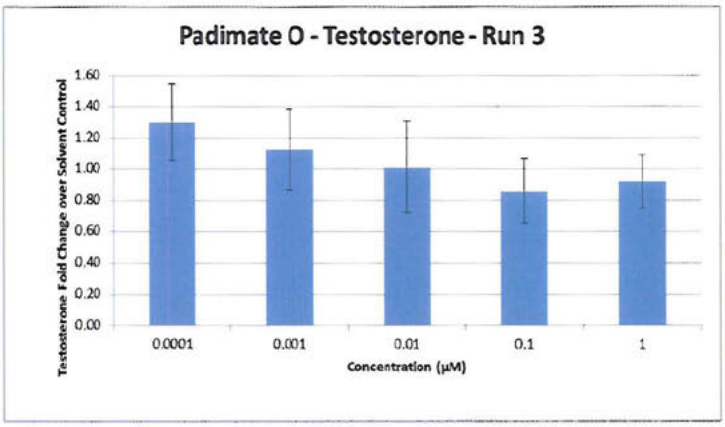
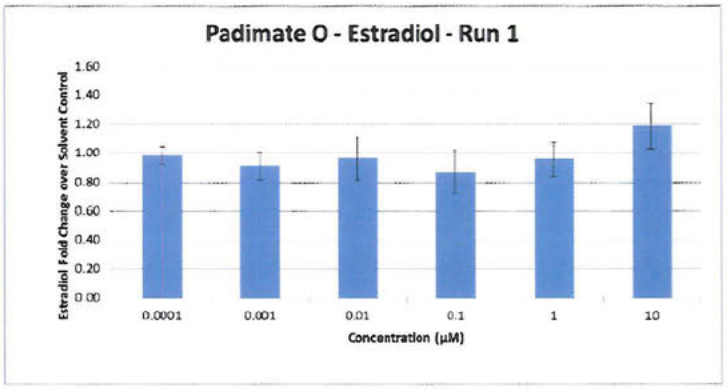
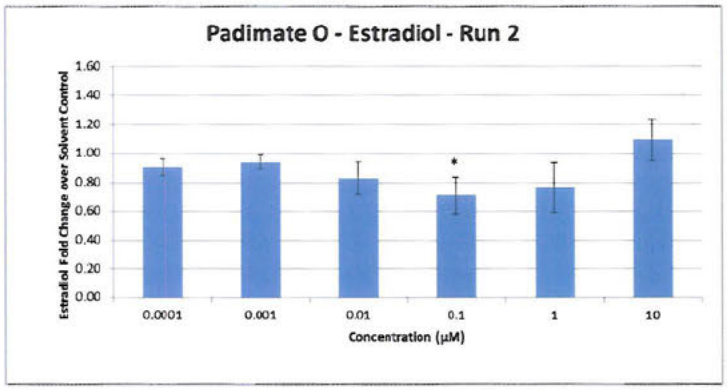


FIGURE 6 Padimate O – Estradiol Fold Change





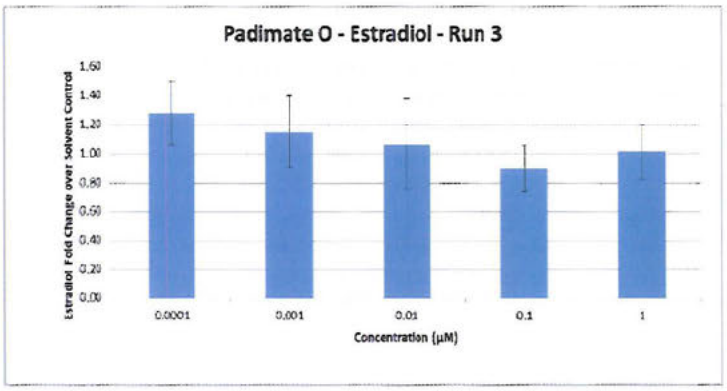
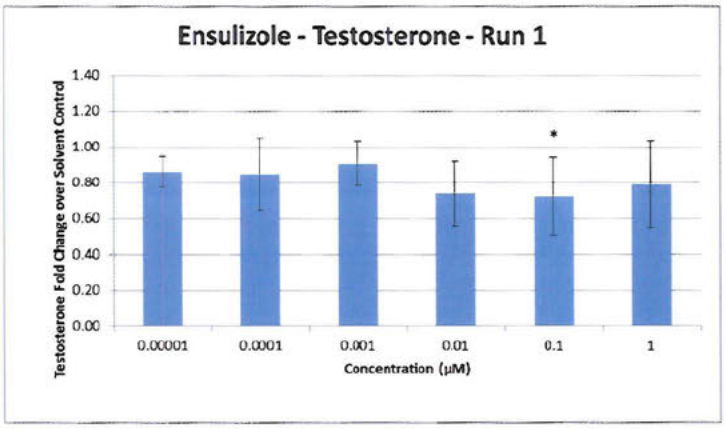
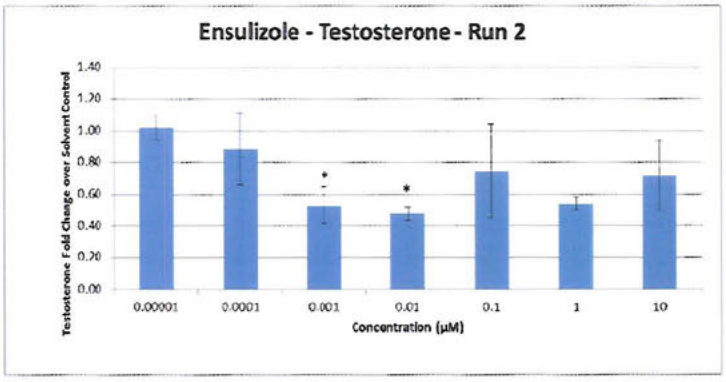


FIGURE 7 Ensulizole – Testosterone Fold Change





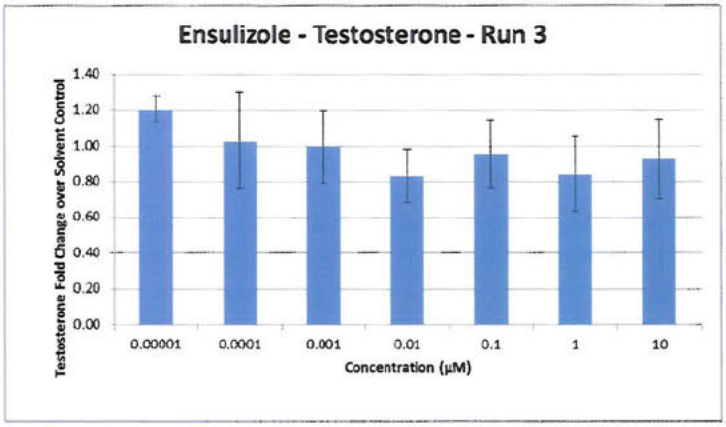
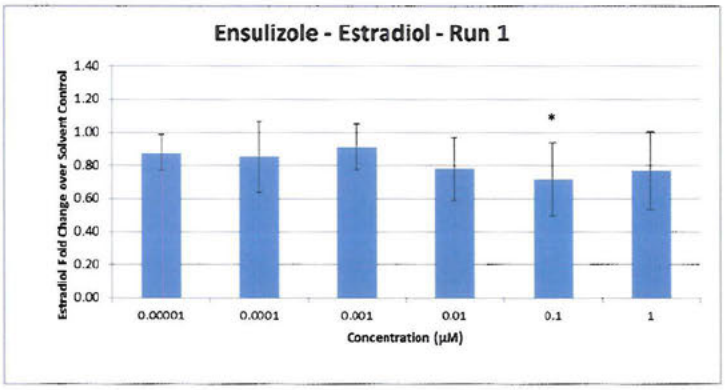
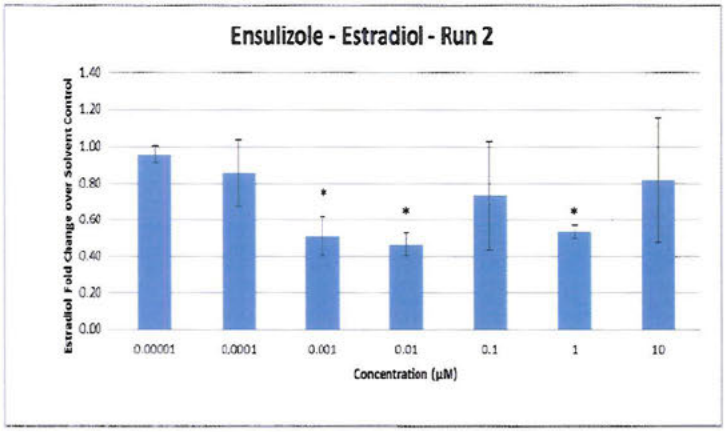


FIGURE 8 Ensulizole – Estradiol Fold Change





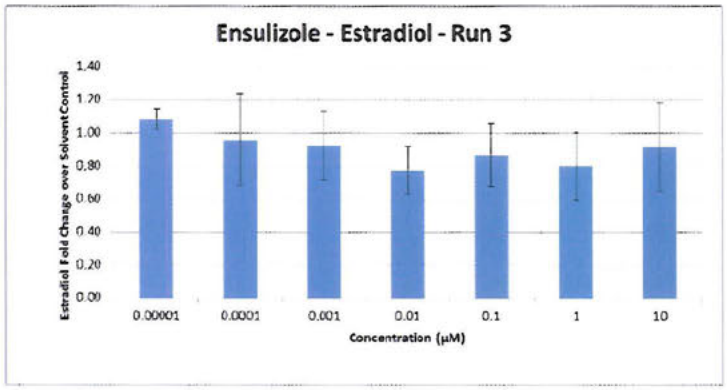
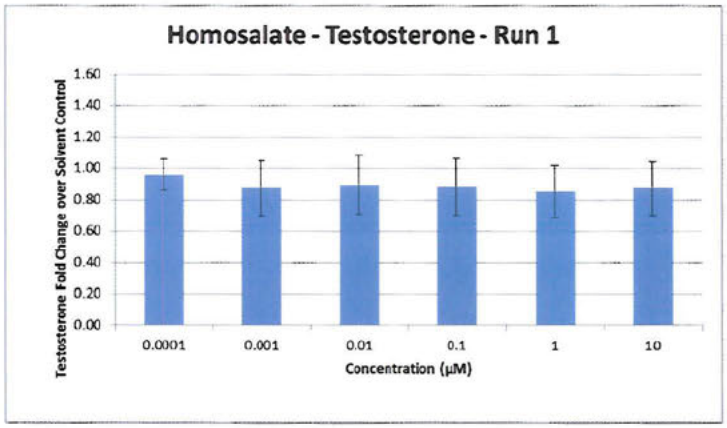
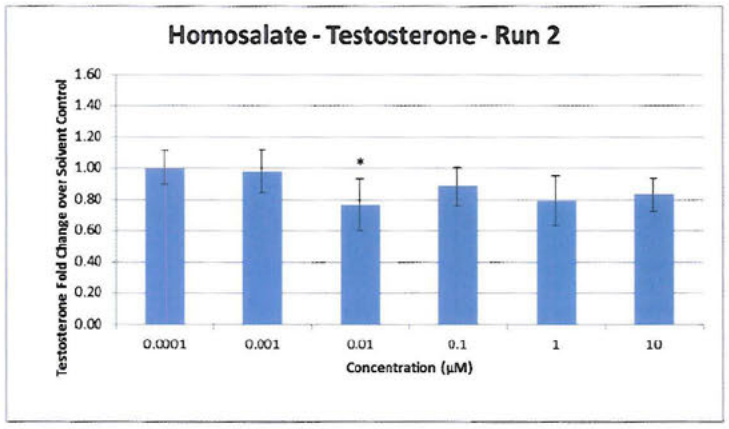


FIGURE 9 Homosalate – Testosterone Fold Change





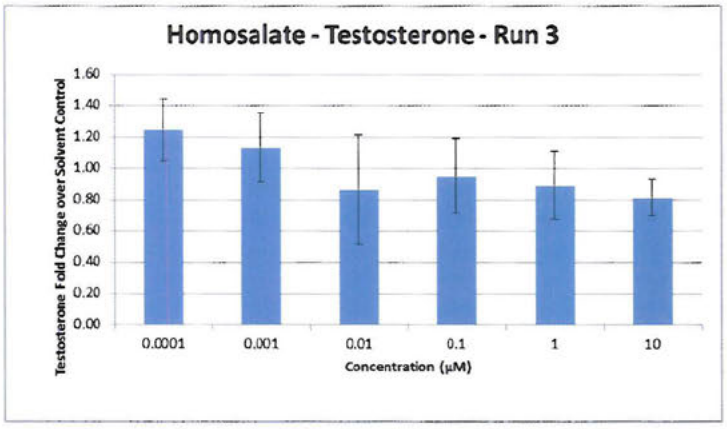
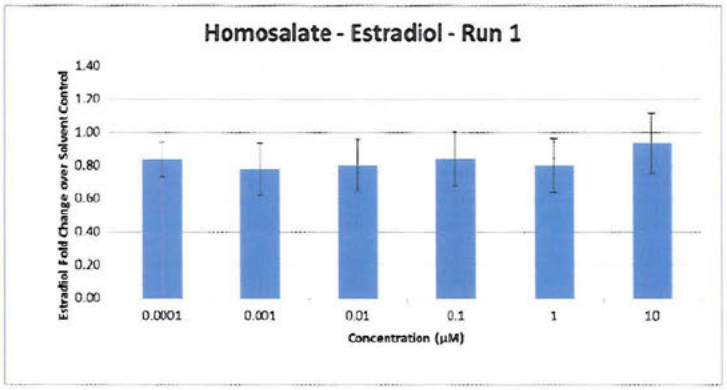
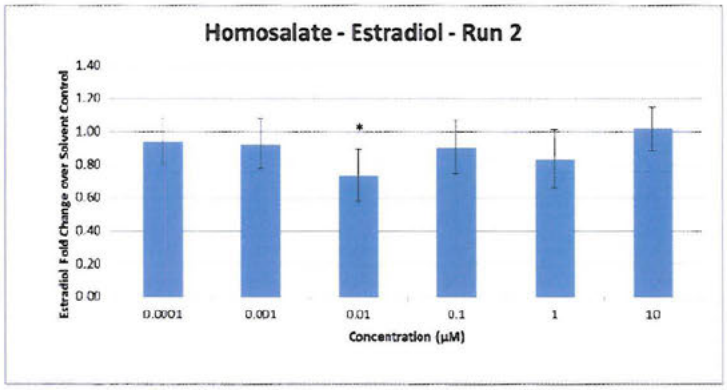


FIGURE 10 Homosalate – Estradiol Fold Change





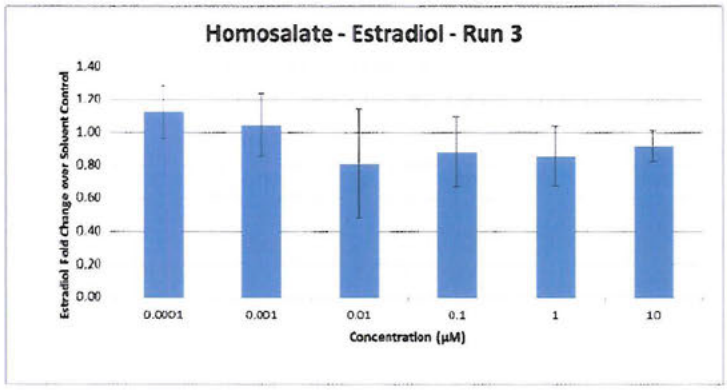
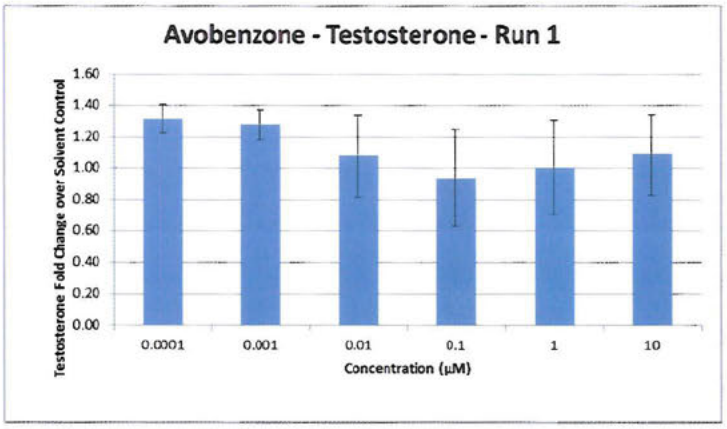
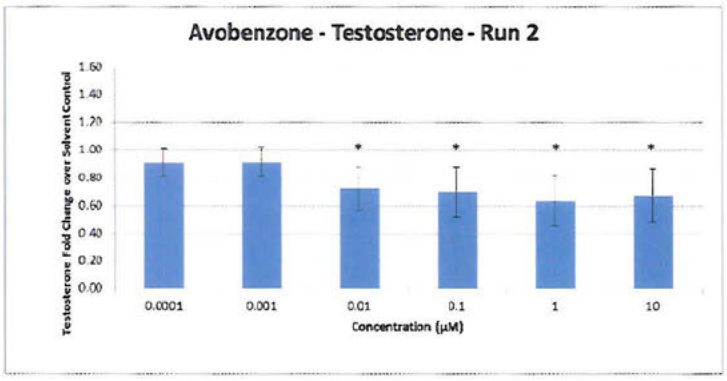


FIGURE 11 Avobenzone – Testosterone Fold Change





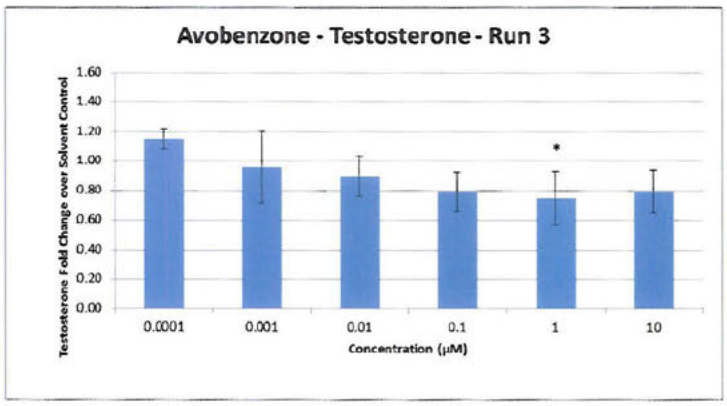
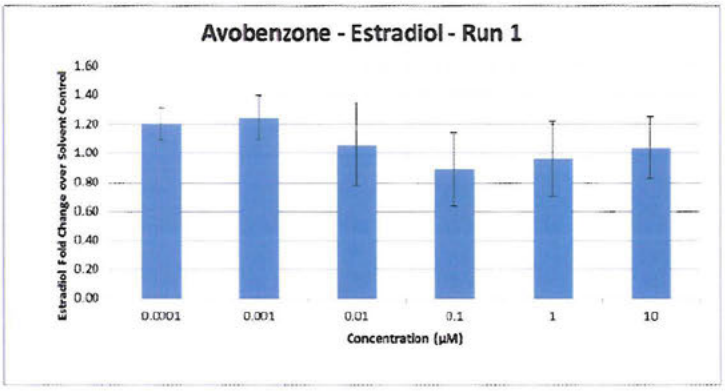
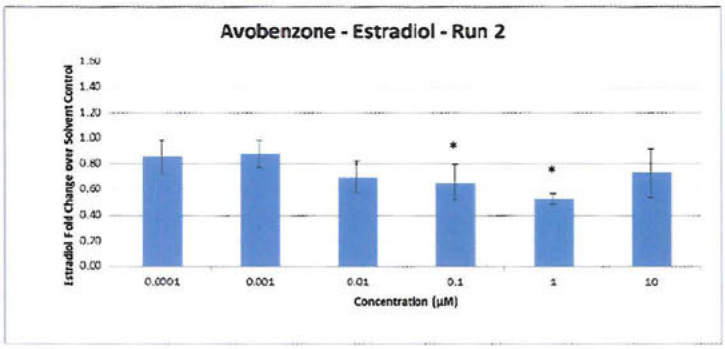
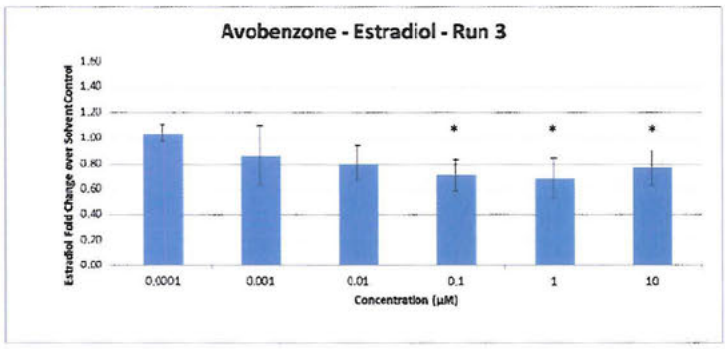


FIGURE 12 Avobenzone – Estradiol Fold Change







APPENDICES SECTION

APPENDIX 1 Data – Padimate O (Run 1)

Testosterone

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	2,651	2,957	2,915	2,632	487.65	1.85E+01
	3,175	1,934	2,161			
0.0001	2,764	2,592	2,857	2,752	148.77	5.41E+00
	2,643	2,671	2,987			
0.001	2,632	2,189	2,631	2,559	269.55	1.05E+01
	2,269	2,835	2,796			
0.01	2,888	3,091	2,821	2,662	427.67	1.61E+01
	2,130	2,114	2,928			
0.1	2,614	2,272	2,456	2,321	397.02	1.71E+01
	1,960	1,788	2,838			
1	2,763	2,556	2,719	2,554	353.90	1.39E+01
	2,123	2,153	3,010			
10	3,044	2,549	2,722	2,725	391.99	1.44E+01
	2,106	2,706	3,227			
100	2,908	2,909	2,364	2,848	256.87	9.02E+00
	3,125	2,827	2,956			

Estradiol

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	65	78	81	68	13.19	1.95E+01
	76	49	56			
0.0001	65	61	72	67	4.22	6.34E+00
	71	64	66			
0.001	62	54	66	62	6.44	1.04E+01
	55	67	68			
0.01	70	74	72	65	9.98	1.53E+01
	53	53	70			
0.1	62	56	65	59	10.29	1.75E+01
	47	49	75			
1	64	65	68	65	7.97	1.23E+01
	57	58	78			
10	86	73	80	80	10.39	1.29E+01
	65	82	95			
100	76	85	70	84	10.14	1.20E+01
	96	96	83			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.187	0.356	0.374	0.334	0.07	2.16E+01
	0.366	0.359	0.359			
0.0001	0.351	0.391	0.338	0.360	0.02	6.04E+00
	0.372	0.337	0.373			
0.001	0.355	0.404	0.344	0.375	0.03	7.30E+00
	0.397	0.351	0.397			
0.01	0.362	0.392	0.347	0.373	0.02	5.89E+00
	0.396	0.352	0.390			
0.1	0.378	0.393	0.358	0.374	0.02	4.50E+00
	0.380	0.349	0.385			
1	0.385	0.387	0.365	0.379	0.01	3.17E+00
	0.379	0.363	0.392			
10	0.376	0.375	0.365	0.368	0.01	3.07E+00
	0.359	0.351	0.380			
100	0.308	0.342	0.349	0.335	0.01	4.33E+00
	0.332	0.337	0.343			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

APPENDIX 1 Data – Padimate O (Run 2)

Testosterone

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	2,915	3,112	3,144	2,992	188.50	6.30E+00
	2,897	2,696	3,186			
0.0001	2,899	3,000	2,771	2,894	116.52	4.03E+00
	2,731	2,991	2,968			
0.001	2,912	2,850	3,026	2,970	103.52	3.49E+00
	2,893	3,009	3,129			
0.01	2,748	2,070	2,837	2,579	367.75	1.43E+01
	2,228	3,024	2,564			
0.1	2,461	2,074	2,047	2,271	372.00	1.64E+01
	2,044	2,953	2,046			
1	2,015	1,833	2,233	2,381	657.02	2.76E+01
	1,825	2,988	3,393			
10	3,196	2,920	2,396	2,777	377.76	1.36E+01
	2,295	2,711	3,145			
100	3,238	2,351	2,595	2,908	477.32	1.64E+01
	3,320	3,444	2,502			

Estradiol

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	59	69	67	62	5.54	8.99E+00
	59	54	61			
0.0001	50	58	60	56	3.41	6.13E+00
	54	57	55			
0.001	58	60	61	58	2.87	4.96E+00
	54	54	60			
0.01	56	44	56	51	6.99	1.37E+01
	43	59	47			
0.1	48	45	37	44	7.82	1.78E+01
	42	57	35			
1	40	39	46	47	10.54	2.24E+01
	37	62	58			
10	80	73	61	68	9.00	1.33E+01
	56	63	72			
100	85	67	65	73	12.07	1.64E+01
	83	84	56			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.335	0.384	0.391	0.375	0.02	5.61E+00
	0.386	0.369	0.386			
0.0001	0.389	0.373	0.384	0.383	0.01	1.51E+00
	0.381	0.388	0.382			
0.001	0.397	0.390	0.383	0.388	0.01	1.30E+00
	0.385	0.388	0.385			
0.01	0.397	0.381	0.390	0.389	0.01	1.46E+00
	0.390	0.393	0.385			
0.1	0.400	0.383	0.405	0.394	0.01	2.34E+00
	0.383	0.399	0.394			
1	0.396	0.380	0.391	0.388	0.01	1.73E+00
	0.382	0.386	0.395			
10	0.406	0.398	0.392	0.396	0.01	1.53E+00
	0.388	0.397	0.396			
100	0.350	0.365	0.346	0.351	0.01	2.43E+00
	0.341	0.356	0.347			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

APPENDIX 1 Data – Padimate O (Run 3)

Testosterone*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	3,936	2,016	1,983	2,554	806.30	3.16E+01
	3,144	2,076	2,169			
0.0001	3,841	3,487	3,391	3,302	625.92	1.90E+01
	2,063	3,494	3,537			
0.001	3,647	2,491	1,746	2,860	663.44	2.32E+01
	3,205	3,142	2,930			
0.01	3,258	1,798	1,928	2,572	752.42	2.93E+01
	2,892	2,015	3,538			
0.1	2,110	1,654	1,656	2,182	533.62	2.45E+01
	2,106	2,518	3,045			
1	2,396	1,967	2,032	2,334	434.70	1.86E+01
	2,141	3,157	2,309			
10	2,712	2,570	2,115	2,241	380.34	1.70E+01
	1,846	1,964	4,091			
100	2,824	2,445	2,027	3,023	780.36	2.58E+01
	2,974	4,031	3,836			

Estradiol

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	NR	37	34	39	10.30	2.63E+01
	57	34	34			
0.0001	56	54	52	50	8.65	1.73E+01
	33	53	53			
0.001	54	43	27	45	9.66	2.15E+01
	52	49	45			
0.01	51	30	33	42	12.09	2.90E+01
	50	30	56			
0.1	33	29	29	35	6.43	1.83E+01
	36	39	45			
1	42	34	36	40	7.36	1.85E+01
	37	54	36			
10	52	52	40	50	14.33	2.86E+01
	36	45	76			
100	69	61	48	73	17.34	2.37E+01
	76	93	91			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.405	0.402	0.409	0.409	0.01	2.05E+00
	0.418	0.421	0.401			
0.0001	0.421	0.416	0.423	0.420	0.00	8.52E-01
	0.425	0.417	0.418			
0.001	0.425	0.401	0.418	0.411	0.01	2.36E+00
	0.405	0.403	0.416			
0.01	0.409	0.409	0.416	0.410	0.00	8.99E-01
	0.405	0.412	0.409			
0.1	0.418	0.404	0.413	0.411	0.01	1.80E+00
	0.408	0.419	0.401			
1	0.415	0.406	0.396	0.411	0.01	2.56E+00
	0.408	0.416	0.427			
10	0.420	0.413	0.422	0.415	0.01	1.81E+00
	0.410	0.421	0.403			
100	0.398	0.396	0.403	0.405	0.02	3.72E+00
	0.411	0.431	0.388			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

*Values in CV% column are highlighted in red if CV is greater than 30%. Values in pg/mL columns are highlighted in red if they were determined to be outliers based on Dixon Q analysis. Values determined to be outliers were excluded from the calculations.

APPENDIX 2 Data – Ensulizole (Run 1)

Testosterone*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	2,891	2,884	3,396	2,994	223.51	7.46E+00
	3,012	3,039	2,743			
0.00001	2,556	2,816	2,822	2,578	247.78	9.61E+00
	2,706	2,311	2,257			
0.0001	2,611	3,072	2,882	2,545	598.50	2.35E+01
	2,873	2,414	1,418			
0.001	2,826	2,294	3,161	2,717	366.97	1.35E+01
	3,097	2,385	2,540			
0.01	1,947	2,987	1,580	2,216	540.97	2.44E+01
	2,745	2,152	1,887			
0.1	1,740	2,955	1,783	2,173	647.28	2.98E+01
	1,626	1,886	3,046			
1	2,672	2,935	1,970	2,372	727.87	3.07E+01
	2,482	1,110	3,062			
10	3,035	3,049	2,765	2,277	761.54	3.34E+01
	1,661	1,319	1,836			

Estradiol*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	65	76	80	72	5.40	7.50E+00
	68	73	69			
0.00001	58	68	70	63	7.73	1.22E+01
	68	66	50			
0.0001	59	73	69	61	15.62	2.54E+01
	69	67	31			
0.001	68	53	76	66	9.70	1.48E+01
	77	64	57			
0.01	50	77	42	56	13.73	2.44E+01
	69	51	48			
0.1	39	72	41	52	15.80	3.04E+01
	40	47	72			
1	63	71	45	56	16.88	3.03E+01
	56	28	71			
10	68	69	65	54	15.44	2.87E+01
	42	35	43			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.304	0.358	0.383	0.358	0.03	7.82E+00
	0.372	0.371	0.357			
0.00001	0.350	0.399	0.350	0.369	0.02	6.34E+00
	0.386	0.344	0.383			
0.0001	0.362	0.407	0.358	0.382	0.02	6.49E+00
	0.392	0.359	0.411			
0.001	0.366	0.405	0.352	0.379	0.02	6.12E+00
	0.391	0.358	0.402			
0.01	0.382	0.393	0.373	0.383	0.02	4.00E+00
	0.389	0.359	0.402			
0.1	0.380	0.386	0.371	0.380	0.01	2.67E+00
	0.381	0.368	0.396			
1	0.383	0.387	0.370	0.380	0.01	2.23E+00
	0.383	0.368	0.387			
10	0.386	0.372	0.378	0.378	0.01	1.64E+00
	0.370	0.383	0.380			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

*Values in CV% column are highlighted in red if CV is greater than 30%. Values in pg/mL columns are highlighted in red if they were determined to be outliers based on Dixon Q analysis. Values determined to be outliers were excluded from the calculations.

APPENDIX 2 Data – Ensulizole (Run 2)

Testosterone*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	3,105	2,885	2,646	2,947	228.92	7.77E+00
	3,282	2,778	2,988			
0.00001	2,918	2,822	3,228	3,000	225.96	7.53E+00
	2,684	3,126	3,221			
0.0001	3,454	1,619	3,063	2,600	662.24	2.55E+01
	2,086	2,633	2,746			
0.001	3,486	1,766	1,490	1,559	342.69	2.20E+01
	1,955	1,044	1,541			
0.01	3,391	1,494	1,368	1,392	129.26	9.29E+00
	1,333	1,221	1,544			
0.1	3,566	2,813	1,595	2,194	871.66	3.97E+01
	1,493	1,377	2,321			
1	3,217	1,412	1,535	1,589	131.20	8.26E+00
	1,756	1,573	1,669			
10	2,494	2,878	1,637	2,109	660.72	3.13E+01
	1,671	1,281	2,694			

Estradiol*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	63	65	55	62	5.15	8.33E+00
	69	57	62			
0.00001	58	59	64	59	2.91	4.93E+00
	56	60	57			
0.0001	67	36	59	53	11.13	2.10E+01
	45	57	53			
0.001	67	37	31	32	6.71	2.13E+01
	38	21	30			
0.01	68	33	28	29	3.87	1.34E+01
	26	24	33			
0.1	73	60	34	45	18.23	4.02E+01
	30	28	47			
1	71	31	35	33	2.15	6.49E+00
	36	31	32			
10	85	61	35	51	21.04	4.15E+01
	39	28	56			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.381	0.391	0.387	0.389	0.01	1.36E+00
	0.389	0.387	0.397			
0.00001	0.432	0.383	0.396	0.398	0.02	4.37E+00
	0.388	0.395	0.394			
0.0001	0.405	0.385	0.387	0.393	0.01	2.00E+00
	0.395	0.387	0.398			
0.001	0.399	0.387	0.401	0.396	0.01	1.41E+00
	0.397	0.401	0.392			
0.01	0.413	0.384	0.398	0.398	0.01	2.64E+00
	0.390	0.406	0.396			
0.1	0.403	0.391	0.400	0.400	0.01	1.54E+00
	0.394	0.408	0.401			
1	0.405	0.387	0.394	0.396	0.01	1.61E+00
	0.391	0.398	0.399			
10	0.408	0.393	0.403	0.399	0.01	1.64E+00
	0.393	0.402	0.393			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

*Values in CV% column are highlighted in red if CV is greater than 30%. Values in pg/mL columns are highlighted in red if they were determined to be outliers based on Dixon Q analysis. Values determined to be outliers were excluded from the calculations.

APPENDIX 2 Data – Ensulizole (Run 3)

Testosterone

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	3,631	3,121	2,343	2,877	668.30	2.32E+01
	2,275	3,534	2,256			
0.00001	3,271	3,308	3,578	3,465	208.02	6.00E+00
	3,260	3,655	3,716			
0.0001	3,823	3,266	3,253	2,952	778.84	2.64E+01
	2,111	3,395	1,865			
0.001	3,154	3,549	3,095	2,862	584.98	2.04E+01
	3,052	2,358	1,963			
0.01	2,984	2,876	1,899	2,396	433.93	1.81E+01
	2,162	2,169	2,286			
0.1	3,637	3,112	2,218	2,741	557.73	2.03E+01
	2,559	2,185	2,736			
1	3,394	1,825	2,210	2,421	601.17	2.48E+01
	2,582	1,821	2,696			
10	3,768	3,130	2,228	2,666	650.61	2.44E+01
	2,116	2,488	2,264			

Estradiol

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	63	55	39	48	12.78	2.65E+01
	37	61	35			
0.00001	50	54	55	52	2.98	5.72E+00
	48	52	54			
0.0001	57	57	53	46	13.44	2.92E+01
	30	52	28			
0.001	47	58	50	44	10.19	2.29E+01
	46	35	30			
0.01	46	46	30	37	6.95	1.87E+01
	36	33	33			
0.1	57	48	33	42	9.31	2.23E+01
	40	33	39			
1	56	31	36	39	9.95	2.58E+01
	42	28	38			
10	65	55	34	44	13.00	2.95E+01
	36	40	34			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.437	0.417	0.424	0.427	0.01	1.58E+00
	0.430	0.430	0.426			
0.00001	0.421	0.417	0.425	0.421	0.00	1.01E+00
	0.426	0.418	0.416			
0.0001	0.415	0.414	0.417	0.415	0.01	1.42E+00
	0.404	0.415	0.422			
0.001	0.425	0.417	0.414	0.420	0.00	9.97E-01
	0.417	0.421	0.423			
0.01	0.426	0.410	0.427	0.420	0.01	1.46E+00
	0.420	0.418	0.421			
0.1	0.420	0.413	0.422	0.415	0.02	3.68E+00
	0.386	0.420	0.430			
1	0.422	0.415	0.423	0.416	0.01	1.84E+00
	0.402	0.419	0.415			
10	0.423	0.408	0.434	0.419	0.01	2.54E+00
	0.407	0.425	0.414			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

APPENDIX 3 Data – Homosalate (Run 1)

Testosterone

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	2,731	3,079	2,862	2,708	401.19	1.48E+01
	2,012	3,054	2,509			
0.0001	2,114	2,599	2,722	2,595	264.76	1.02E+01
	2,876	2,524	2,732			
0.001	2,265	2,415	2,843	2,367	473.50	2.00E+01
	1,732	1,996	2,951			
0.01	2,807	2,358	2,595	2,421	505.54	2.09E+01
	3,046	1,668	2,051			
0.1	3,044	2,486	2,297	2,390	483.17	2.02E+01
	2,618	1,575	2,320			
1	2,974	2,587	2,013	2,313	452.69	1.96E+01
	2,537	1,847	1,919			
10	2,703	3,130	1,935	2,368	460.75	1.95E+01
	2,286	2,024	2,130			
100	2,749	3,129	2,837	2,644	535.98	2.03E+01
	2,190	1,811	3,148			

Estradiol

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	73	86	81	73	11.32	1.56E+01
	58	79	60			
0.0001	47	64	66	61	7.75	1.27E+01
	68	60	61			
0.001	56	61	70	57	11.50	2.03E+01
	41	46	66			
0.01	67	59	62	58	11.50	1.97E+01
	72	41	49			
0.1	77	66	55	61	12.11	1.98E+01
	69	43	57			
1	74	70	53	58	11.97	2.06E+01
	60	45	46			
10	77	92	59	68	13.32	1.95E+01
	64	59	60			
100	93	114	97	92	16.67	1.82E+01
	73	71	102			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.363	0.211	0.384	0.344	0.07	1.90E+01
	0.366	0.365	0.373			
0.0001	0.353	0.390	0.352	0.371	0.02	5.90E+00
	0.394	0.348	0.388			
0.001	0.354	0.405	0.347	0.380	0.03	8.10E+00
	0.405	0.356	0.414			
0.01	0.366	0.388	0.356	0.379	0.02	6.07E+00
	0.400	0.356	0.409			
0.1	0.374	0.388	0.368	0.380	0.02	4.16E+00
	0.389	0.360	0.403			
1	0.366	0.383	0.375	0.380	0.01	2.89E+00
	0.386	0.373	0.397			
10	0.346	0.366	0.368	0.369	0.02	4.11E+00
	0.377	0.365	0.392			
100	0.198	0.316	0.348	0.319	0.06	1.92E+01
	0.341	0.347	0.363			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

APPENDIX 3 Data – Homosalate (Run 2)

Testosterone

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	2,843	2,581	3,101	3,034	437.09	1.44E+01
	2,905	2,916	3,858			
0.0001	3,156	3,107	3,149	3,032	329.89	1.09E+01
	3,239	3,177	2,365			
0.001	3,122	3,186	3,486	2,966	409.94	1.38E+01
	2,361	2,609	3,031			
0.01	3,278	2,266	1,873	2,330	504.06	2.16E+01
	2,063	2,059	2,444			
0.1	3,110	3,093	2,669	2,682	370.60	1.38E+01
	2,181	2,642	2,396			
1	3,092	2,826	1,800	2,406	480.57	2.00E+01
	2,047	2,369	2,303			
10	3,099	2,447	2,429	2,529	316.87	1.25E+01
	2,642	2,173	2,386			
100	2,858	2,472	2,138	2,318	339.45	1.46E+01
	1,972	2,015	2,451			

Estradiol

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	65	51	59	62	6.35	1.02E+01
	63	64	70			
0.0001	54	61	63	59	8.88	1.52E+01
	68	63	43			
0.001	59	69	67	58	9.49	1.65E+01
	47	48	56			
0.01	64	48	38	46	10.02	2.18E+01
	40	37	48			
0.1	71	66	52	56	10.00	1.77E+01
	44	53	52			
1	66	65	41	52	10.98	2.12E+01
	43	50	46			
10	78	63	63	63	8.13	1.28E+01
	65	55	56			
100	86	81	68	75	10.65	1.42E+01
	59	72	85			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.375	0.426	0.402	0.400	0.02	4.07E+00
	0.400	0.396	0.402			
0.0001	0.424	0.395	0.403	0.401	0.01	2.97E+00
	0.394	0.396	0.393			
0.001	0.423	0.398	0.400	0.402	0.01	2.63E+00
	0.394	0.399	0.397			
0.01	0.424	0.394	0.373	0.396	0.02	4.15E+00
	0.391	0.396	0.399			
0.1	0.411	0.400	0.394	0.398	0.01	1.93E+00
	0.398	0.389	0.393			
1	0.412	0.394	0.395	0.397	0.01	2.01E+00
	0.389	0.394	0.400			
10	0.396	0.394	0.393	0.390	0.01	1.79E+00
	0.377	0.393	0.388			
100	0.356	0.353	0.361	0.348	0.01	2.92E+00
	0.337	0.344	0.337			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

APPENDIX 3 Data – Homosalate (Run 3)

Testosterone*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	3,096	3,118	1,867	2,699	507.13	1.88E+01
	2,895	2,289	2,930			
0.0001	3,735	3,276	3,702	3,350	531.89	1.59E+01
	2,316	3,519	3,548			
0.001	3,613	3,214	3,056	3,040	596.09	1.96E+01
	3,020	1,914	3,425			
0.01	3,554	3,516	1,902	2,320	948.27	4.09E+01
	1,622	1,742	1,581			
0.1	3,460	3,115	2,020	2,557	648.31	2.54E+01
	1,745	2,605	2,398			
1	3,448	2,057	1,894	2,394	584.39	2.44E+01
	2,284	2,671	2,014			
10	2,510	1,643	2,272	2,185	306.27	1.40E+01
	2,147	2,119	2,422			
100	3,459	2,541	1,913	2,580	550.24	2.13E+01
	2,540	2,137	2,891			

Estradiol*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	54	52	33	46	8.46	1.84E+01
	51	39	47			
0.0001	55	54	56	52	7.58	1.46E+01
	36	54	56			
0.001	53	53	49	48	8.78	1.83E+01
	48	31	54			
0.01	59	55	29	37	15.33	4.12E+01
	28	28	24			
0.1	55	48	31	41	9.92	2.44E+01
	30	41	37			
1	54	35	33	39	8.42	2.15E+01
	37	44	32			
10	49	36	43	42	4.30	1.02E+01
	42	40	43			
100	85	72	47	68	13.51	1.99E+01
	65	60	78			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.401	0.398	0.415	0.408	0.01	2.03E+00
	0.407	0.420	0.409			
0.0001	0.422	0.418	0.412	0.417	0.01	1.23E+00
	0.421	0.421	0.410			
0.001	0.414	0.409	0.418	0.415	0.00	1.02E+00
	0.421	0.413	0.413			
0.01	0.426	0.424	0.418	0.417	0.01	1.74E+00
	0.408	0.411	0.413			
0.1	0.434	0.407	0.426	0.415	0.01	3.12E+00
	0.407	0.414	0.400			
1	0.426	0.406	0.418	0.414	0.01	2.54E+00
	0.399	0.423	0.409			
10	0.407	0.397	0.404	0.406	0.01	1.95E+00
	0.401	0.420	0.409			
100	0.398	0.352	0.384	0.370	0.02	4.86E+00
	0.366	0.367	0.353			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

*Values in CV% column are highlighted in red if CV is greater than 30%. Values in pg/mL columns are highlighted in red if they were determined to be outliers based on Dixon Q analysis. Values determined to be outliers were excluded from the calculations.

APPENDIX 4 Data – Avobenzone (Run 1)

Testosterone*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	2,171	1,658	2,197	2,211	615.43	2.78E+01
	1,424	2,877	2,935			
0.0001	2,499	3,006	3,020	2,903	201.21	6.93E+00
	3,007	2,925	2,961			
0.001	2,451	2,839	2,825	2,819	212.55	7.54E+00
	2,911	3,102	2,788			
0.01	2,290	2,659	2,849	2,379	581.34	2.44E+01
	1,679	3,065	1,730			
0.1	2,417	1,209	1,690	2,071	686.23	3.31E+01
	1,918	1,976	3,217			
1	2,405	1,726	1,539	2,222	650.74	2.93E+01
	1,722	2,906	3,030			
10	2,469	1,622	1,884	2,400	569.07	2.37E+01
	2,774	2,482	3,172			
100	1,688	2,248	1,988	2,026	196.76	9.71E+00
	1,958	2,147	2,124			

Estradiol*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	52	43	NR	57	18.58	3.27E+01
	36	77	75			
0.0001	56	70	73	68	6.40	9.35E+00
	72	71	67			
0.001	61	71	77	71	8.58	1.21E+01
	82	74	60			
0.01	55	69	77	60	16.28	2.70E+01
	45	77	39			
0.1	58	29	50	51	14.24	2.81E+01
	50	45	72			
1	58	44	43	55	14.52	2.66E+01
	39	74	69			
10	60	42	49	59	12.03	2.03E+01
	72	62	71			
100	13	15	13	15	1.60	1.07E+01
	15	17	16			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.356	0.201	0.378	0.338	0.07	2.00E+01
	0.362	0.367	0.363			
0.0001	0.353	0.398	0.348	0.370	0.02	5.99E+00
	0.390	0.351	0.382			
0.001	0.344	0.404	0.343	0.374	0.03	8.64E+00
	0.398	0.347	0.408			
0.01	0.375	0.391	0.346	0.375	0.02	5.67E+00
	0.387	0.352	0.397			
0.1	0.376	0.395	0.354	0.378	0.02	4.56E+00
	0.388	0.361	0.393			
1	0.363	0.383	0.367	0.370	0.01	3.23E+00
	0.370	0.353	0.384			
10	0.329	0.339	0.326	0.331	0.01	2.24E+00
	0.330	0.322	0.341			
100	0.146	0.213	0.226	0.210	0.03	1.54E+01
	0.216	0.224	0.235			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

*Values in CV% column are highlighted in red if CV is greater than 30%. Values in pg/mL columns are highlighted in red if they were determined to be outliers based on Dixon Q analysis. Values determined to be outliers were excluded from the calculations.

APPENDIX 4 Data – Avobenzone (Run 2)

Testosterone

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	3,399	3,323	2,240	3,121	461.86	1.48E+01
	3,084	3,148	3,534			
0.0001	3,435	2,711	2,733	2,834	303.72	1.07E+01
	2,704	2,593	2,826			
0.001	3,223	3,147	2,615	2,853	325.19	1.14E+01
	2,361	2,935	2,840			
0.01	1,732	1,844	1,979	2,269	492.00	2.17E+01
	2,961	2,442	2,654			
0.1	2,013	1,573	1,869	2,171	567.90	2.62E+01
	1,829	2,731	3,013			
1	1,769	1,715	1,690	1,987	573.14	2.89E+01
	1,650	1,959	3,135			
10	2,211	1,497	1,786	2,104	600.97	2.86E+01
	1,548	2,599	2,982			
100	1,080	783	808	1,123	290.64	2.59E+01
	1,523	1,258	1,287			

Estradiol*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	66	66	47	62	7.26	1.17E+01
	65	64	63			
0.0001	68	54	45	53	7.93	1.50E+01
	51	49	50			
0.001	64	60	46	54	6.75	1.24E+01
	48	55	53			
0.01	35	36	38	43	7.88	1.83E+01
	56	47	45			
0.1	38	31	36	40	8.50	2.11E+01
	35	49	52			
1	32	33	32	32	2.37	7.31E+00
	30	36	60			
10	52	34	37	45	11.98	2.66E+01
	33	56	60			
100	13	11	11	16	4.67	2.97E+01
	22	19	19			

MTT

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.382	0.413	0.398	0.395	0.01	2.60E+00
	0.396	0.391	0.392			
0.0001	0.402	0.386	0.400	0.394	0.01	1.71E+00
	0.387	0.396	0.391			
0.001	0.401	0.408	0.399	0.404	0.01	2.44E+00
	0.393	0.421	0.399			
0.01	0.408	0.409	0.401	0.401	0.01	1.64E+00
	0.391	0.399	0.400			
0.1	0.417	0.399	0.401	0.402	0.01	2.27E+00
	0.391	0.407	0.396			
1	0.403	0.391	0.404	0.394	0.01	2.04E+00
	0.383	0.391	0.392			
10	0.352	0.347	0.343	0.343	0.01	2.35E+00
	0.329	0.339	0.347			
100	0.267	0.265	0.292	0.271	0.01	4.12E+00
	0.273	0.266	0.261			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

*Values in CV% column are highlighted in red if CV is greater than 30%. Values in pg/mL columns are highlighted in red if they were determined to be outliers based on Dixon Q analysis. Values determined to be outliers were excluded from the calculations.

APPENDIX 4 Data – Avobenzone (Run 3)

Testosterone*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	3,217	3,279	2,221	3,218	522.10	1.62E+01
	3,362	3,497	3,735			
0.0001	3,984	3,517	3,529	3,692	209.36	5.67E+00
	3,602	3,933	3,589			
0.001	3,753	3,587	1,750	3,072	790.29	2.57E+01
	3,215	3,058	3,351			
0.01	2,329	2,608	3,260	2,877	443.04	1.54E+01
	2,511	3,216	3,339			
0.1	2,545	2,327	1,955	2,538	424.76	1.67E+01
	2,660	3,246	2,493			
1	2,187	2,318	2,115	2,391	587.84	2.46E+01
	2,180	1,979	3,570			
10	2,188	2,188	2,383	2,539	461.64	1.82E+01
	2,310	2,809	3,357			
100	1,201	1,298	1,382	1,499	379.02	2.53E+01
	1,205	2,146	1,763			

Estradiol*

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	63	64	41	58	8.87	1.52E+01
	59	64	60			
0.0001	68	60	59	60	3.99	6.61E+00
	58	60	56			
0.001	60	59	27	50	13.81	2.75E+01
	55	51	60			
0.01	36	43	54	47	7.98	1.71E+01
	41	55	53			
0.1	42	38	32	41	7.45	1.80E+01
	44	54	38			
1	36	38	37	40	9.15	2.29E+01
	36	35	59			
10	38	39	42	45	7.96	1.78E+01
	41	51	58			
100	15	14	16	17	3.56	2.10E+01
	15	24	18			

MTT*

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
SC	0.399	0.406	0.414	0.408	0.01	1.36E+00
	0.413	0.408	0.405			
0.0001	0.417	0.419	0.422	0.419	0.00	7.79E-01
	0.424	0.415	0.419			
0.001	0.417	0.415	0.423	0.417	0.00	9.03E-01
	0.413	0.418	1.860			
0.01	0.423	0.407	0.395	0.413	0.01	2.72E+00
	0.412	0.414	0.426			
0.1	0.423	0.405	0.412	0.412	0.01	1.69E+00
	0.405	0.409	0.416			
1	0.414	0.405	0.399	0.402	0.01	1.86E+00
	0.392	0.399	0.405			
10	0.360	0.354	0.353	0.356	0.00	8.82E-01
	0.360	0.354	0.357			
100	0.308	0.308	0.308	0.303	0.01	2.58E+00
	0.288	0.301	0.304			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

*Contamination was observed in one well at the 0.001 µM exposure concentration. This concentration was excluded from the analysis.

APPENDIX 5 QC Plate Data – MTT

Run 1

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
Blank	0.353	0.274	0.383	0.355	0.04	1.15E+01
	0.372	0.376	0.372			
Background	0.323	0.377	0.319	0.348	0.03	8.37E+00
	0.371	0.322	0.375			
Vehicle	0.352	0.374	0.386	0.372	0.01	3.38E+00
	0.365	0.384	0.372			
Vehicle + MeOH	0.020	0.028	0.019	0.023	0.00	1.84E+01
	0.028	0.021	0.020			
Forskolin 1 µM	0.388	0.397	0.384	0.392	0.01	1.63E+00
	0.392	0.391	0.402			
Forskolin 10 µM	0.391	0.402	0.388	0.399	0.01	3.21E+00
	0.407	0.388	0.420			
Prochloraz 0.1 µM	0.366	0.396	0.351	0.377	0.02	5.11E+00
	0.389	0.362	0.395			
Prochloraz 1 µM	0.372	0.394	0.355	0.376	0.02	4.06E+00
	0.390	0.364	0.383			

Run 2

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
Blank	0.238	0.369	0.322	0.339	0.05	1.59E+01
	0.374	0.353	0.380			
Background	0.367	0.352	0.370	0.361	0.01	2.55E+00
	0.349	0.370	0.360			
Vehicle	0.395	0.367	0.385	0.382	0.01	2.35E+00
	0.383	0.383	0.381			
Vehicle + MeOH	0.022	0.024	0.027	0.024	0.00	7.13E+00
	0.025	0.024	0.023			
Forskolin 1 µM	0.404	0.370	0.387	0.393	0.01	3.17E+00
	0.401	0.396	0.397			
Forskolin 10 µM	0.402	0.389	0.407	0.399	0.01	1.72E+00
	0.404	0.393	0.400			
Prochloraz 0.1 µM	0.370	0.372	0.380	0.374	0.01	1.44E+00
	0.379	0.378	0.367			
Prochloraz 1 µM	0.392	0.370	0.375	0.380	0.01	2.25E+00
	0.377	0.388	0.375			

Run 3

	Absorbance Values					
Dose [uM]	a	b	c	Avg.	SD	CV%
Blank	0.420	0.402	0.412	0.413	0.01	1.85E+00
	0.420	0.419	0.407			
Background	0.399	0.403	0.410	0.406	0.00	1.03E+00
	0.406	0.409	0.408			
Vehicle	0.446	0.409	0.433	0.423	0.01	3.53E+00
	0.411	0.428	0.412			
Vehicle + MeOH	0.022	0.025	0.029	0.025	0.00	1.02E+01
	0.027	0.026	0.023			
Forskolin 1 µM	0.437	0.440	0.432	0.436	0.01	1.27E+00
	0.433	0.443	0.428			
Forskolin 10 µM	0.454	0.438	0.455	0.443	0.01	2.52E+00
	0.434	0.448	0.428			
Prochloraz 0.1 µM	0.438	0.412	0.413	0.421	0.01	2.30E+00
	0.416	0.424	0.420			
Prochloraz 1 µM	0.429	0.402	0.427	0.420	0.01	2.97E+00
	0.416	0.435	0.411			

MTT values are subtracted values ($Absorbance_{570} - Absorbance_{650}$).

APPENDIX 6 QC Plate Data - Testosterone

Testosterone – Run 1

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
Background	1568	1529	1072	1455	265.09	1.82E+01
Background	1570	1203	1786			
Blank	2958	2872	1850	2809	520.46	1.85E+01
Blank	2737	3037	3401			
DMSO	2775	3062	2514	2955	318.72	1.08E+01
DMSO	2892	3023	3465			
Forskolin 1 µM	4943	6104	4823	5834	975.33	1.67E+01
Forskolin 1 µM	5554	6096	7483			
Forskolin 10 µM	7830	7268	6837	7508	1010.66	1.35E+01
Forskolin 10 µM	6454	7320	9338			
Prochloraz 0.1 µM	1528	2290	2258	2340	579.88	2.48E+01
Prochloraz 0.1 µM	2259	2366	3342			
Prochloraz 1 µM	787	1051	1042	1008	126.54	1.25E+01
Prochloraz 1 µM	973	1027	1171			

Testosterone – Run 2

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
Background	1372	1266	1173	1347	109.28	8.11E+00
Background	1474	1422	1374			
Blank	2166	2586	2745	2678	298.66	1.12E+01
Blank	3018	2641	2910			
DMSO	2670	2931	2697	2753	133.58	4.85E+00
DMSO	2617	2912	2692			
Forskolin 1 µM	3356	4016	4459	4102	404.71	9.87E+00
Forskolin 1 µM	4362	4073	4346			
Forskolin 10 µM	4930	5703	5787	5572	337.65	6.06E+00
Forskolin 10 µM	5602	5536	5876			
Prochloraz 0.1 µM	1574	1794	2189	2055	307.54	1.50E+01
Prochloraz 0.1 µM	2290	2119	2366			
Prochloraz 1 µM	934	858	877	929	52.27	5.63E+00
Prochloraz 1 µM	947	963	996			

Testosterone – Run 3

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
Background	1558	1480	1450	1513	67.49	4.46E+00
Background	1457	1510	1625			
Blank	3929	3371	3344	3225	427.93	1.33E+01
Blank	2844	2747	3117			
DMSO	3163	3680	3284	3203	566.80	1.77E+01
DMSO	3359	2118	3614			
Forskolin 1 µM	4988	5508	5490	5139	411.77	8.01E+00
Forskolin 1 µM	5449	4899	4497			
Forskolin 10 µM	7251	4119	6216	6133	1091.99	1.78E+01
Forskolin 10 µM	6250	6040	6925			
Prochloraz 0.1 µM	3141	2851	2463	2802	244.02	8.71E+00
Prochloraz 0.1 µM	2667	2697	2993			
Prochloraz 1 µM	1406	1176	1197	1177	129.80	1.10E+01
Prochloraz 1 µM	1055	1049	1182			

APPENDIX 6 QC Plate Data – Estradiol

Estradiol – Run 1

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
Background	NQ	NQ	NQ	#DIV/0!	#DIV/0!	#DIV/0!
Background	NQ	NQ	NQ			
Blank	77	77	51	71	10.91	1.54E+01
Blank	69	82	69			
DMSO	67	71	66	68	3.29	4.84E+00
DMSO	73	67	64			
Forskolin 1 µM	320	453	357	418	66.02	1.58E+01
Forskolin 1 µM	421	471	485			
Forskolin 10 µM	780	756	704	758	50.65	6.68E+00
Forskolin 10 µM	696	789	826			
Prochloraz 0.1 µM	40	62	65	61	10.79	1.78E+01
Prochloraz 0.1 µM	62	64	72			
Prochloraz 1 µM	20	30	28	26	3.32	1.27E+01
Prochloraz 1 µM	25	27	26			

Estradiol – Run 2

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
Background	NQ	NQ	NQ	#DIV/0!	#DIV/0!	#DIV/0!
Background	NQ	NQ	NQ			
Blank	41	50	56	54	9.64	1.78E+01
Blank	70	50	57			
DMSO	53	61	53	56	3.71	6.65E+00
DMSO	53	59	55			
Forskolin 1 µM	305	410	482	436	70.10	1.61E+01
Forskolin 1 µM	487	473	461			
Forskolin 10 µM	702	856	900	862	85.00	9.86E+00
Forskolin 10 µM	917	939	856			
Prochloraz 0.1 µM	36	42	48	48	7.13	1.50E+01
Prochloraz 0.1 µM	50	52	56			
Prochloraz 1 µM	22	21	20	21	1.33	6.31E+00
Prochloraz 1 µM	20	22	23			

Estradiol – Run 3

	pg/ml					
Dose [uM]	a	b	c	Avg.	SD	CV%
Background	16	12	10	12	2.27	1.93E+01
Background	10	NQ	11			
Blank	67	70	62	60	7.68	1.29E+01
Blank	52	52	56			
DMSO	57	63	57	55	9.12	1.65E+01
DMSO	55	38	61			
Forskolin 1 µM	566	724	714	650	85.65	1.32E+01
Forskolin 1 µM	723	642	529			
Forskolin 10 µM	1241	795	1280	1176	188.64	1.60E+01
Forskolin 10 µM	1258	1207	1276			
Prochloraz 0.1 µM	64	58	51	58	4.80	8.24E+00
Prochloraz 0.1 µM	60	54	62			
Prochloraz 1 µM	32	29	30	28	2.86	1.01E+01
Prochloraz 1 µM	26	24	29			

APPENDIX 7 Solubility Data

Run 1 – 0HR

	Ensulizole		Avobenzone		Homosalate		Padimate O	
	Avg	StDev	Avg	StDev	Avg	StDev	Avg	StDev
Stock 1	1411.7	888.2	1809.3	210.4	338.3	22.0	227.7	10.1
Stock 2	310.7	150.7	387.0	19.3	247.0	27.7	215.7	49.7
Stock 3	207.0	15.6	229.7	43.4	225.3	26.3	165.3	21.7
Stock 4	233.7	65.1	338.7	199.1	230.3	16.6	183.0	45.4
Stock 5	241.0	101.7	360.3	193.3	192.3	24.2	160.3	20.3
Stock 6	243.0	49.6	305.3	115.6	330.0	182.8	161.7	51.0
Stock 7	360.3	154.5	220.0	57.0	223.7	68.2	148.0	15.5

Run 2 – 0HR

	Ensulizole		Avobenzone		Homosalate		Padimate O	
	Avg	StDev	Avg	StDev	Avg	StDev	Avg	StDev
Stock 1	88.0	11.3	2172.3	283.1	123.7	11.1	244.7	22.0
Stock 2	122.7	29.7	110.7	4.2	132.7	35.1	242.0	96.9
Stock 3	105.3	16.4	120.0	6.0	97.3	6.1	142.7	13.2
Stock 4	181.7	138.0	133.7	61.8	141.3	60.5	154.3	11.7
Stock 5	134.3	46.5	122.0	28.6	97.3	8.3	148.7	42.1
Stock 6	112.0	13.1	209.7	63.5	92.0	12.2	195.0	158.5
Stock 7	162.7	64.9	101.7	17.0	105.3	1.5	211.3	129.9

Run 3 – 0HR

	Ensulizole		Avobenzone		Homosalate		Padimate O	
	Avg	StDev	Avg	StDev	Avg	StDev	Avg	StDev
Stock 1	133.3	19.6	3310.0	78.8	173.0	18.4	166.0	42.5
Stock 2	137.3	7.0	155.3	8.0	145.0	29.6	129.3	18.0
Stock 3	187.7	54.9	136.3	23.8	114.0	8.9	246.0	245.1
Stock 4	133.3	42.2	118.3	18.1	150.0	11.5	142.3	42.8
Stock 5	145.7	10.2	133.7	17.5	138.0	7.5	125.3	5.5
Stock 6	228.3	162.5	206.3	90.8	158.7	70.9	148.7	87.8
Stock 7	272.3	231.9	134.0	8.9	149.7	52.4	129.0	11.5

APPENDIX 8 Statistics – Padimate O (Run 1)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9526	0.0797	Shapiro-Wilk's (residuals)	0.9732	0.4215
Levene's Test	1.4589	0.2209	Levene's Test	1.8170	0.1242
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	Results Below		Dunnett's Test (original data)	Results Below	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	N/A	N/A	Shapiro-Wilk's (log residuals)	N/A	N/A
Levene's Test (transformed data)	N/A	N/A	Levene's Test (transformed data)	N/A	N/A
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	N/A		Kruskal-Wallis (Dunn)	N/A	

Testosterone – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.0001 - 0	0.9835
10 - 0	0.9955
0.01 - 0	1.0000
0.001 - 0	0.9988
1 - 0	0.9983
0.1 - 0	0.5059

Estradiol – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
10 - 0	0.1028
0.0001 - 0	1.0000
0.01 - 0	0.9956
1 - 0	0.9911
0.001 - 0	0.7721
0.1 - 0	0.4064

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 8 Statistics – Padimate O (Run 2)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9621	0.1761	Shapiro-Wilk's (residuals)	0.9705	0.3421
Levene's Test	5.5248	0.0004	Levene's Test	3.4783	0.0084
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	N/A		Dunnett's Test (original data)	Results Below	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	0.9636	0.1992	Shapiro-Wilk's (log residuals)	0.9768	0.5411
Levene's Test (transformed data)	5.9341	0.0002	Levene's Test (transformed data)	3.8982	0.0044
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)		
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	Results Below		Kruskal-Wallis (Dunn)	Results Below	

Testosterone – Kruskal-Wallis (Dunn) Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.001 - 0	1.0000
0.0001 - 0	1.0000
10 - 0	1.0000
0.01 - 0	0.4423
1 - 0	0.2052
0.1 - 0	0.0438*

Estradiol – Kruskal-Wallis (Dunn) Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
10 - 0	1.0000
0.001 - 0	1.0000
0.0001 - 0	1.0000
0.01 - 0	0.2303
1 - 0	0.1190
0.1 - 0	0.0144*

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 8 Statistics – Padimate O (Run 3)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9857	0.9124	Shapiro-Wilk's (residuals)	0.9880	0.9615
Levene's Test	1.1293	0.3664	Levene's Test	1.1016	0.3809
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	Results Below		Dunnett's Test (original data)	Results Below	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	N/A	N/A	Shapiro-Wilk's (log residuals)	N/A	N/A
Levene's Test (transformed data)	N/A	N/A	Levene's Test (transformed data)	N/A	N/A
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	N/A		Kruskal-Wallis (Dunn)	N/A	

Testosterone – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.0001 - 0	0.1924
0.001 - 0	0.8792
0.01 - 0	1.0000
1 - 0	0.9647
0.1 - 0	0.7777

Estradiol – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.0001 - 0	0.2042
0.001 - 0	0.7225
0.01 - 0	0.9854
1 - 0	1.0000
0.1 - 0	0.9220

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 9 Statistics – Ensulizole (Run 1)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9726	0.4023	Shapiro-Wilk's (residuals)	0.9736	0.4334
Levene's Test	2.1981	0.0665	Levene's Test	1.7108	0.1476
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	Results Below		Dunnett's Test (original data)	Results Below	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	N/A	N/A	Shapiro-Wilk's (log residuals)	N/A	N/A
Levene's Test (transformed data)	N/A	N/A	Levene's Test (transformed data)	N/A	N/A
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	N/A		Kruskal-Wallis (Dunn)	N/A	

Testosterone – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.001 - 0	0.8571
0.00001 - 0	0.5434
0.0001 - 0	0.4688
1 - 0	0.1769
0.01 - 0	0.0590
0.1 - 0	0.0421*

Estradiol – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.001 - 0	0.9049
0.00001 - 0	0.7087
0.0001 - 0	0.5243
0.01 - 0	0.1680
1 - 0	0.1424
0.1 - 0	0.0480*

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 9 Statistics – Ensulizole (Run 2)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9740	0.4018	Shapiro-Wilk's (residuals)	0.9318	0.0109
Levene's Test	5.8472	0.0001	Levene's Test	5.9317	0.0001
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	N/A		Dunnett's Test (original data)	N/A	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	0.9769	0.5007	Shapiro-Wilk's (log residuals)	0.9675	0.2340
Levene's Test (transformed data)	6.4004	0.0001	Levene's Test (transformed data)	6.5865	0.0000
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	Results Below		Kruskal-Wallis (Dunn)	Results Below	

Testosterone – Kruskal-Wallis Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.00001 - 0	1.0000
0.0001 - 0	1.0000
0.1 - 0	0.5252
10 - 0	0.4321
1 - 0	0.0512
0.001 - 0	0.0322*
0.01 - 0	0.0023*

Estradiol – Kruskal-Wallis Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.00001 - 0	1.0000
0.0001 - 0	1.0000
10 - 0	1.0000
0.1 - 0	0.4321
1 - 0	0.0340*
0.001 - 0	0.0151*
0.01 - 0	0.0025*

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 9 Statistics – Ensulizole (Run 3)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9734	0.3427	Shapiro-Wilk's (residuals)	0.9805	0.6004
Levene's Test	1.7385	0.1276	Levene's Test	2.7455	0.0200
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	Results Below		Dunnett's Test (original data)		
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	N/A	N/A	Shapiro-Wilk's (log residuals)	0.9779	0.4936
Levene's Test (transformed data)	N/A	N/A	Levene's Test (transformed data)	2.7381	0.0203
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	N/A		Kruskal-Wallis (Dunn)	Results Below	

Testosterone – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.00001 - 0	0.3590
0.0001 - 0	1.0000
0.001 - 0	1.0000
0.1 - 0	0.9991
10 - 0	0.9838
1 - 0	0.6204
0.01 - 0	0.5665

Estradiol – Kruskal-Wallis (Dunn) Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.00001 - 0	1.0000
0.0001 - 0	1.0000
0.001 - 0	1.0000
10 - 0	1.0000
0.1 - 0	1.0000
1 - 0	0.8539
0.01 - 0	0.3184

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 10 Statistics – Homosalate (Run 1)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9806	0.6848	Shapiro-Wilk's (residuals)	0.9690	0.3063
Levene's Test	0.5774	0.7457	Levene's Test	0.5405	0.7737
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	Results Below		Dunnett's Test (original data)	Results Below	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	N/A	N/A	Shapiro-Wilk's (log residuals)	N/A	N/A
Levene's Test (transformed data)	N/A	N/A	Levene's Test (transformed data)	N/A	N/A
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	N/A		Kruskal-Wallis (Dunn)	N/A	

Testosterone – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.0001 - 0	0.9952
0.01 - 0	0.7374
0.1 - 0	0.6536
10 - 0	0.5923
0.001 - 0	0.5889
1 - 0	0.4447

Estradiol – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
10 - 0	0.9689
0.1 - 0	0.3240
0.0001 - 0	0.3094
0.01 - 0	0.1530
1 - 0	0.1449
0.001 - 0	0.0896

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 10 Statistics – Homosalate (Run 2)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9724	0.3967	Shapiro-Wilk's (residuals)	0.9645	0.2136
Levene's Test	0.3154	0.9246	Levene's Test	0.6093	0.7211
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	Results Below		Dunnett's Test (original data)	Results Below	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	N/A	N/A	Shapiro-Wilk's (log residuals)	N/A	N/A
Levene's Test (transformed data)	N/A	N/A	Levene's Test (transformed data)	N/A	N/A
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	N/A		Kruskal-Wallis (Dunn)	N/A	

Testosterone – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.0001 - 0	1.0000
0.001 - 0	0.9997
0.1 - 0	0.4933
10 - 0	0.1704
1 - 0	0.0575
0.01 - 0	0.0272*

Estradiol – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
10 - 0	0.9999
0.0001 - 0	0.9639
0.001 - 0	0.9038
0.1 - 0	0.7747
1 - 0	0.2356
0.01 - 0	0.0224*

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 10 Statistics – Homosalate (Run 3)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9777	0.5729	Shapiro-Wilk's (residuals)	0.9793	0.6359
Levene's Test	1.9372	0.1021	Levene's Test	2.5605	0.0367
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	Results Below		Dunnett's Test (original data)	N/A	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	N/A	N/A	Shapiro-Wilk's (log residuals)	0.9758	0.5050
Levene's Test (transformed data)	N/A	N/A	Levene's Test (transformed data)	2.9465	0.0196
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	N/A		Kruskal-Wallis (Dunn)	Results Below	

Testosterone – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.0001 - 0	0.2874
0.001 - 0	0.8431
0.1 - 0	0.9973
1 - 0	0.8961
0.01 - 0	0.7770
10 - 0	0.5149

Estradiol – Kruskal-Wallis (Dunn) Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.0001 - 0	0.7924
0.001 - 0	1.0000
10 - 0	1.0000
0.1 - 0	1.0000
1 - 0	1.0000
0.01 - 0	1.0000

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 11 Statistics – Avobenzone (Run 1)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9677	0.2748	Shapiro-Wilk's (residuals)	0.9655	0.2434
Levene's Test	2.3733	0.0499	Levene's Test	2.2219	0.0647
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	N/A		Dunnett's Test (original data)	Results Below	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	0.9752	0.4848	Shapiro-Wilk's (log residuals)	N/A	N/A
Levene's Test (transformed data)	2.6905	0.0297	Levene's Test (transformed data)	N/A	N/A
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	Results Below		Kruskal-Wallis (Dunn)	N/A	

Testosterone – Kruskal-Wallis (Dunn) Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.0001 - 0	0.1266
0.001 - 0	0.5971
0.01 - 0	1.0000
10 - 0	1.0000
1 - 0	1.0000
0.1 - 0	1.0000

Estradiol – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.001 - 0	0.3251
0.0001 - 0	0.5106
0.01 - 0	0.9956
10 - 0	0.9995
1 - 0	0.9996
0.1 - 0	0.9246

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 11 Statistics – Avobenzone (Run 2)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9712	0.3633	Shapiro-Wilk's (residuals)	0.9814	0.7291
Levene's Test	1.0513	0.4098	Levene's Test	2.7429	0.0278
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	Results Below		Dunnett's Test (original data)	N/A	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	N/A	N/A	Shapiro-Wilk's (log residuals)	0.9757	0.5184
Levene's Test (transformed data)	N/A	N/A	Levene's Test (transformed data)	3.8908	0.0046
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	N/A		Kruskal-Wallis (Dunn)	Results Below	

Testosterone – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.001 - 0	0.8486
0.0001 - 0	0.8073
0.01 - 0	0.0227*
0.1 - 0	0.0094*
10 - 0	0.0050*
1 - 0	0.0016*

Estradiol – Kruskal-Wallis (Dunn) Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.001 - 0	1.0000
0.0001 - 0	1.0000
10 - 0	0.1410
0.01 - 0	0.0595
0.1 - 0	0.0168*
1 - 0	0.0002*

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 11 Statistics – Avobenzone (Run 3)

Testosterone	Test Statistic:	p-value:	Estradiol	Test Statistic:	p-value:
Original Data Set:			**Original Data Set:**		
Shapiro-Wilk's (residuals)	0.9748	0.4858	Shapiro-Wilk's (residuals)	0.9663	0.2593
Levene's Test	0.6839	0.6637	Levene's Test	0.8788	0.5208
Parametric Test (original data):			**Parametric Test (original data):**		
Dunnett's Test (original data)	Results Below		Dunnett's Test (original data)	Results Below	
Log Transformation:			**Log Transformation:**		
Shapiro-Wilk's (log residuals)	N/A	N/A	Shapiro-Wilk's (log residuals)	N/A	N/A
Levene's Test (transformed data)	N/A	N/A	Levene's Test (transformed data)	N/A	N/A
Parametric Test (transformed data):			**Parametric test (transformed data):**		
Dunnett's Test (transformed data)	N/A		Dunnett's Test (transformed data)	N/A	
Non-parametric Test (original data):			**Non-parametric Test (original data):**		
Kruskal-Wallis (Dunn)	N/A		Kruskal-Wallis (Dunn)	N/A	

Testosterone – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.0001 - 0	0.4076
0.001 - 0	0.9933
0.01 - 0	0.7155
10 - 0	0.1158
0.1 - 0	0.1148
1 - 0	0.0379*

Estradiol – Dunnett's Test Comparison of Treatment Groups to Solvent Controls:

Concentration (µM)	p-value:
0.0001 - 0	0.9974
0.001 - 0	0.4637
0.01 - 0	0.1190
10 - 0	0.0482*
0.1 - 0	0.0096*
1 - 0	0.0046*

*Denotes statistical significance ($p \leq 0.05$).

APPENDIX 12 Deviation Form

Form #: SOP-1003-F-1.2

CeeTox
In vitro models to predict toxicity

Deviation and Investigation

Study Number (if applicable): 9070-100794STER

SOP Number (if applicable): N/A

Equipment Serial Number (if applicable): N/A

Date of Reporting: 10 April 2013 Reporting Associate: [redacted]

Date of Occurrence: 02 April 2013, 10 April 2013 Associate Involved: [redacted]

Description of Deviation:

Ensulizole was insoluble in DMSO at the 200 mM stock concentration specified in section 9 of the protocol. Because of the solubility issue, ensulizole was prepared in DMSO at 20 mM. This was the Stock 1 solution for this test substance. As a result, the final exposure concentrations for ensulizole were 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 µM.

Signature [redacted] Date: 10 April 2013

Reporting Associate

Type of Deviation (determined by Study Director/Principal Investigator/Management):

☐ SOP Deviation ☒ Protocol Deviation ☐ GLP Deviation ☐ Facility Deviation ☐ No Deviation

Summary of Deviation Investigation by SD/PI/Test Facility Management/Designee:
This change is a deviation from the study protocol. Section 9 of the protocol specifies that a 200 mM stock solution will be prepared as the highest stock solution concentration. Because of the insolubility, a 20 mM stock solution was prepared. Protocol Section 15 and Table 3 specify the final exposure concentrations for the test substances. The final exposure concentrations for ensulizole were modified because of the reduction in stock solution concentration.

Action Taken and Determination of Impact on Study Data and/or Facility Compliance:
A protocol amendment will be prepared to incorporate this change for remaining assay runs. The change will not impact the integrity of the study data.

Signature [redacted] Date: 10 April 2013

SD/PI/Management Acknowledgement

Signature [redacted] Date: 10 Apr 2013

Quality Assurance Review

Standard Operating Procedure Page 1 of 2

CeeTox
In vitro models to predict toxicity

Deviation and Investigation

If study specific, sponsor notified on: 10 April 2013 By: [redacted]

See Attached Documentation (email documentation is sufficient) ☒

APPENDIX 13 Certificates of Analysis

9070 - 100794 STER

SIGMA-ALDRICH

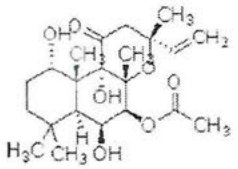
sigma-aldrich.com

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

Certificate of Analysis

Product Name:
Forskolin - from Coleus forskohlii, BioReagent, for molecular biology, ≥98%

Product Number:	F3917
Lot Number:	SLBB5661V
Brand:	SIGMA
CAS Number:	66575-29-9
MDL Number:	MFCD00082317
Formula:	$C_{22}H_{34}O_7$
Formula Weight:	410.50 g/mol
Quality Release Date:	08 MAR 2012



Test	Specification	Result
Appearance (Color)	White to Off-White	White
Appearance (Form)	Powder	Powder
Solubility (Color)	Colorless to Faint Yellow	Colorless
Solubility (Turbidity) 50 mg/mL in EtOH	Clear	Clear
Infrared spectrum	Conforms to Structure	Conforms
Suitability	Suitable	Suitable
Inhibits Interleukin 2 (IL-2) production in jurkat cells		
Purity (HPLC)	≥ 98 %	98 %

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

9070-100794STER

SIGMA-ALDRICH

CERTIFICATE OF ANALYSIS

Sigma-Aldrich Laborchemikalien GmbH D-30918 Seelze
Telefon: +49 5137 8238-150

Seelze, 17.05.2010/120592/10/08510

Order-No. :
Customer-No.:

Order-Code:

Quantity:

Production Date: 22.Apr.2010
Expiry Date: 22.Apr.2015

Article/Product: 45631 Batch : SZBA112X

Prochloraz PESTANAL®

Reference Material (RM)

1. General Information

Formula: C15H16Cl3N3O2 Molar mass: 376.67 g/Mole
CAS-No.: [67747-09-5] Recomm. storage temp.: roomtemp.
Usage : Fungicide

The estimated uncertainty of a single measurement of the assay can be expected to be 0.5 % relative (confidence level = 95%, n= 6) whereby the assay measurements are calculated by 100% minus found impurities.

2. Batch Analysis

Identity (NMR)	complying	
Assay (HPLC)	99.1	area %
Melting range	45.2-46.1	°C
Water (Karl Fischer)	0.09	%
Date of Analysis	12.May.2010	

3. Advice and Remarks

- The minimum shelf life is based on the current knowledge and holds only for proper storage conditions in the originally closed flasks/ packages
- Whenever the container is opened for removal of aliquot portions of the substance, the person handling the substance must ensure, that the integrity of the substance is maintained and proper records of all its handlings are kept. Special care has to be taken to avoid any contamination or adulteration of the substance
- We herewith confirm that the delivery is effected according to the technical delivery conditions agreed.
- Particular properties of the products or the suitability for a particular area of application are not assured.
- We guarantee a proper quality within our General Conditions of Sales.

Sigma-Aldrich Laborchemikalien GmbH
Quality Management SA-LC

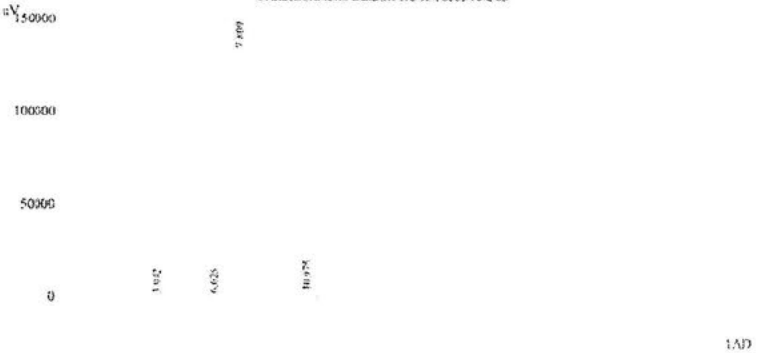
This document was produced electronically and is valid without a signature

9070-100794STER

HPLC-Method

Article	: Prochloraz
Article-No	: 45631
Batch	: SZHA112X
Column	: L=250mm, ID=4,6mm; Supelcosil LC-18 5µm
Fluent	: 60 % Acetonitrile 40 % Water + 0,28% Na2HPO4
Flow	: 1,4ml/min
Detector	: UV-225nm
Injection-Volume	: 10µl
Sample-Preparation	: 0,2mg/ml
Linearity	: checked
Evaluation	: Normalisation (uncorrected)
Operator	: [redacted]

Chromatogram
C:\LabSolutions\Data\HPLC II\100510C03



1 AD1 r

Peak Table

AD1 ch1

Peak#	Ret. Time	Area	Area %
1	3.942	2731	0.022
2	6.625	6855	0.055
3	7.809	12292434	99.138
4	10.975	37298	0.285
Total		12339318	100.000

RTI INTERNATIONAL
NTP Analytical Chemistry Services
3040 Cornwallis Road • PO Box 12194 • Research Triangle Park, NC 27709-2194 • USA
Telephone 919.541.6720 or 919.541.5975 • Fax 919.485.2650 • www.rti.org

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

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

ENSULIZOLE

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:
[redacted]
09-05-12
Date
Task Leader

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

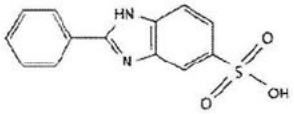
Submitted to:

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

ENSULIZOLE

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

STRUCTURE



MOL. WT.
274.30

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

EXECUTIVE SUMMARY

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

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: Ensulizole

Task Type: Chemical Reanalysis

Chem Task Number: CHEM11786

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

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Sample Preparation Inspection for HPLC Analysis	05/15/12	05/22/12
Data & Report Audit	08/24/12 & 08/28/12	08/28/12

Prepared by:

[redacted] 9-5-12
Quality Assurance Specialist Date

Reviewed by:

[redacted] 9/5/12
Quality Assurance Specialist Date

turning knowledge into practice

TABLE OF CONTENTS

Figures

ENSULIZOLE

1.0 INTRODUCTION

The objective of this work was to determine the purity and verify the identity of ensulizole to the current studies being conducted at RTI International. To accomplish this objective, a bulk chemical reanalysis was performed. The identity of the chemical was confirmed by FTIR and its purity assessed by LC.

2.0 CHEMICAL ANALYSIS

An aliquot of the bulk sample of ensulizole was received at the analytical laboratory on March 27, 2012 for chemical reanalysis (RTI log 082010-C-15). The aliquot was stored at room temperature. A frozen reference (RTI log 082010-C-05) sample was received at the analytical laboratory on May 10, 2012 and was stored at freezer temperature.

3.0 CONFIRMATION OF IDENTITY - INFRARED SPECTROMETRY (IR)

3.1 IR Parameters

System	Thermo Nicolet 6700 FTIR
Software	Omnic, Ver. 7.3
Method	KBr pellet, scan 4000 - 400 cm^{-1}

3.2 Results

Bulk Sample Frequency (1/cm)	Frozen Reference Sample Frequency (1/cm)	Assignment
3367	3372	N-H stretch
3059-2725	3059-2725	O-H, N-H, C-H stretch
1633, 1568	1630, 1567	C=C, C=N stretch
1368	1368	C-N stretch
1176	1176	C-C, SO_2 stretch
1028	1028	N-H bend
780	777	C-H, N-H bend
631	630	S-O stretch

The observed spectrum for the bulk sample matched the spectrum of the frozen reference sample, and is consistent with the structure of ensulizole (as reported in the characterization protocols development task CHEM11291). Figure 1 shows the IR spectra for the bulk and frozen samples.

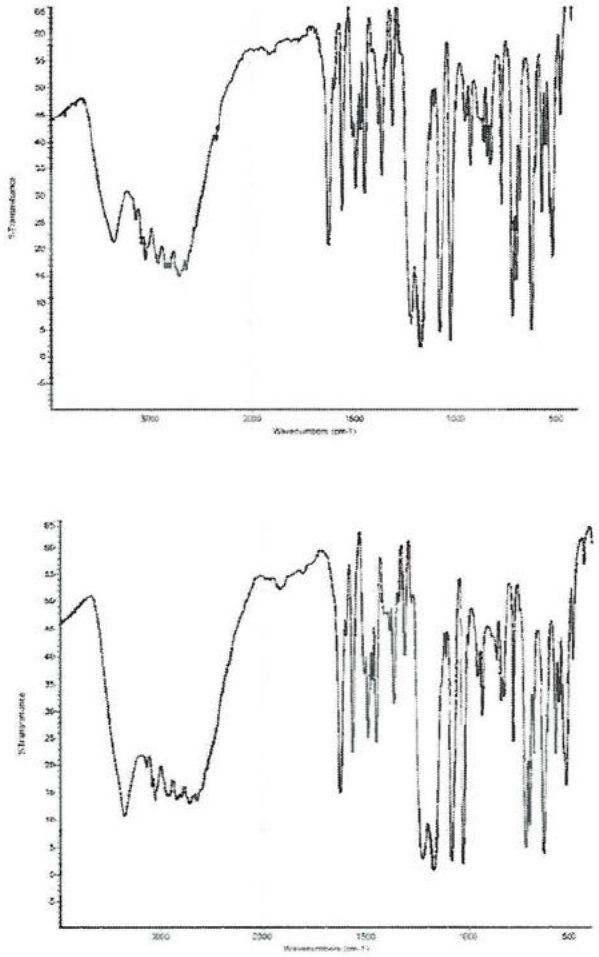


Figure 1: Infrared Spectrum of Ensulizole Frozen Reference (top spectrum) and Bulk Sample (bottom spectrum)

2

4.0 DETERMINATION OF PURITY - LIQUID CHROMATOGRAPHY

This section describes the liquid chromatographic method used to estimate sample purity.

4.1 Preparation of Internal Standard (IS) Solution

A stock solution of IS was prepared by weighing 500 mg of padimate O and transferring it into a 10-mL volumetric flask. The IS was diluted to volume with mobile phase B (methanol with 0.1% formic acid). The flask was mixed by inversion. A working IS solution (WIS) was prepared as a 1 mL to 1 L dilution with mobile phase B and mixing by inversion, yielding 0.050 mg/mL working IS.

4.2 Bulk Sample and Frozen Reference Standard Solution Preparation

Triplicate solutions of the reference standard and bulk samples were prepared by transferring approximately 25 mg of compound to individual 100-mL volumetric flasks and diluting to volume with WIS and mixing by inversion. All samples were transferred to autosampler vials and analyzed by liquid chromatography.

4.3 Analysis

LC Parameters

System	Waters Alliance 2695
Software	Empower 2; Build 2154
Column	Waters XBridge C18 3.5 µm, 100 x 2.1 mm, guard column, 5 µm 2.1 x 10 mm
Column Temp	40 °C
Mobile Phases	A: 0.1% formic acid in water B: 0.1% formic acid in methanol
Flow Rate	0.25 mL/min
Gradient	Hold 90 % A for 0.67 min., 90% A to 90% B in 10 min., hold 90% B for 10 min., 90% B to 90% A in 3 min., hold 90% A for 5 min.
Injection Volume - Solvent	2 µL - Mobile Phase B
Retention Time (min)	Ensulizole - 5.73 min Padimate O (IS) - 16.59 min
Detector	Waters 2996 PDA, 312 nm

The suitability of the system was evaluated, and the results are shown below.

Parameter	Result	Criteria	Pass/Fail
Capacity Factor, k	2.8	$2 \geq k \leq 12$	Pass
Tailing Factor, T	1.2	$0.5 \geq T \leq 2.0$	Pass
Column Efficiency, N	29,000	$N \geq 6{,}000$ plates	Pass

4.4 Results

Calculations based on a major peak comparison technique gave the results shown in the following table.

RTI Log No.	Chemical	RRF[a]	Mean RRF[a] (%RSD)	Percent Relative Purity[b]
082010-C-15	Analytical Replicate #1	3.072	3.046 (0.82)	99.6
	Analytical Replicate #2	3.022		
	Analytical Replicate #3	3.045		
082010-C-05	Reference Replicate #1	3.034	3.057 (0.81)	–
	Reference Replicate #2	3.083		
	Reference Replicate #3	3.054		

[a] RRF = Relative Response Factor; normalized to sample concentration.

[b] Relative Purity = (Mean RRF, bulk/Mean RRF, ref.) x 100.

Based on the chromatographic results, the bulk sample had not significantly changed as compared to the frozen reference, and no significant impurities were observed. Typical chromatograms are shown in Figure 2.

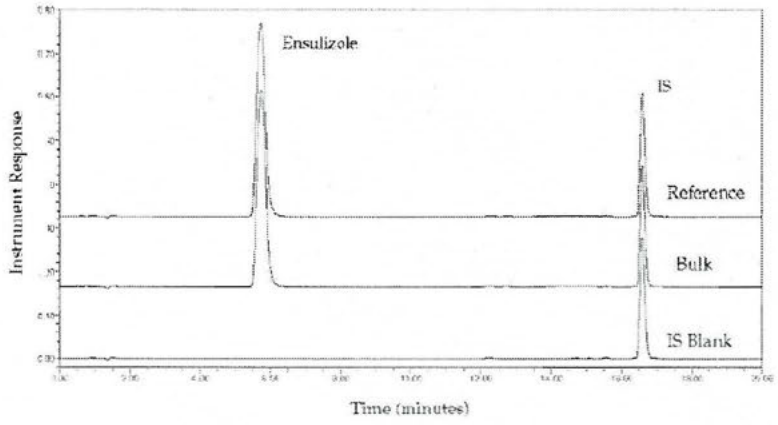


Figure 2: Example Liquid Chromatograms of Ensulizole Reference and Bulk Sample, and a Blank

5.0 REFERENCE

RTI International report "Ensulizole, Characterization Protocols Development (CHEM11291), January 9, 2012.

6.0 ACKNOWLEDGMENTS

Personnel contributing to this task: [redacted].

This PDF File is an Exact Copy of the Report
Signature: [redacted]
Date: 2-16-12

Analytical Chemistry Services for the NTP
NIEHS Contract No. HHSN273201100001C
MRI Project No.: 110730
NTP ChemTask No.: CHEM10985

Chemical Comprehensive Analysis Final Report

Avobenzone

Chemical Comprehensive Analysis of Avobenzone

MRI Assignment No.: 2003

February 16, 2012

Prepared by:

[redacted]
Study Director

Approved by:

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

Reviewed by:

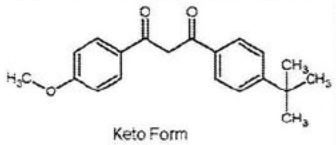
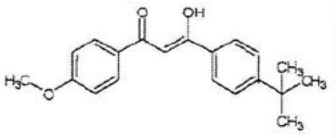
[redacted]
Group Leader

Submitted to:

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

Chemical Comprehensive Analysis of Avobenzone

Chemical Information

CAS No.: 70356-09-1 MRI Assignment No.: 2003 ChemTask No. CHEM10965 Program Supported: TOX Analysis Dates: 2/11/11 to 12/14/11 Interim Result Date(s): 2/25/11, 4/7/11, 5/17/11	Lot No.: L802809 MRI Assigned Batch No.: 01 Amount Received: 20 Kg Sample Receipt Date: 1/5/11 Appearance: Off white to yellowish crystalline powder per CoA; confirmed by visual observation Supplier: Universal Preserv-A-Chem Inc. Supplier Purity: 98.30% per CoA Storage conditions (at Analytical Lab): Ambient, protected from light
 Keto Form  Enol Form (predominant)	Mol. Wt.: 310.39 Mol. Formula: $C_{20}H_{22}O_3$

Executive Summary

The purpose of this assignment was to perform a chemical comprehensive analysis for avobenzone, Lot No. L802809, received from Universal Preserv-A-Chem Inc. Based on the results, the identity of the test article was confirmed to be avobenzone, with a purity of approximately 98.5%. Evaluation by gas chromatography with flame ionization detection of samples stored at various temperatures indicated avobenzone is stable when stored for 2 weeks, protected from light, at temperatures up to approximately 60°C. Nuclear magnetic resonance spectroscopic analysis of these samples, as well as samples exposed to light for 1 week, detected some conversion of enol to keto form under elevated temperature and light exposure.

The chemical comprehensive analysis included identity confirmation using infrared (IR) and nuclear magnetic resonance (NMR) spectroscopy, residual solvent analysis for volatile content using gas chromatography (GC)/headspace analysis, ultraviolet/visible (UV/Vis) spectroscopy, water content using Karl Fischer titration, elemental analysis, determination of melting point, and log P, differential scanning calorimetry (DSC), and chromatographic profiling using gas chromatography (GC) with flame ionization detection (FID). Additionally, gas chromatography/mass spectrometry (GC/MS) was performed to confirm identity of the test article.

Spectra obtained for the test article using IR and NMR spectroscopy techniques were consistent with reference spectra and the proposed structure for the enol form of the test article. One absorbance maximum was observed using ultraviolet/visible spectroscopy: 358 nm, ϵ_{max} = 36241 ± 186(s). Analysis using GC/MS with electron capture ionization provided confirmation of identity based on the molecular ion (310 Da) observed, as well as comparison to a reference spectrum.

Water content determined by Karl Fischer was 0.223 ± 0.008(s) %. Elemental analysis determined 77.36% carbon, 7.39% hydrogen, and 0.02% nitrogen compared to expected values of 77.39 carbon, 7.15% hydrogen, and no nitrogen. The observed melting point range was 83.0° to 85.5°C (literature values of 83.5°C and 81° to 86°C). The determined log P was 3.10.

Differential scanning calorimetry was performed, and the observed melting point range was consistent with the melting point range from the MSDS. The results indicated a purity of 98.8 ± 0.5(d) %. Chromatographic profiling, using GC with a DB-5 column and FID, indicated 98.7% purity, with seven reportable impurities totaling 1.26% relative to the total peak area. GC/headspace analysis indicated residual solvent peak responses for methanol and cis-1,2-dichloroethene, but they were not present at levels greater than the Class 2 Mixture A Standard. There were no other Class 1 or Class 2 solvents observed to be present in the test article.

Accelerated stability was performed using GC with FID to evaluate possible degradation of the test article. The test variability limit (TVL), which is statistically determined, established that in order to be statistically significant at the 95% confidence level, the loss or gain under ambient, refrigerated, or elevated storage conditions must be greater than 3.8% relative to the sample under the frozen storage condition. The maximum variance from the frozen storage condition was +0.7%, observed for the sample stored at approximately 60°C. Using the TVL criteria,

avobenzone is stable when stored for 2 weeks as the bulk chemical, protected from light, at temperatures up to approximately 60°C. An additional evaluation using ^{1}H-NMR spectroscopy of the accelerated stability samples and stability samples exposed to light exhibited decreased enol/keto ratios of the –OH and $-CH_2$ functional groups for the samples stored at 60°C, as well as samples exposed to fluorescent or mercury/xenon lighting. This indicates some conversion of the enol to the keto form.

Quality Assurance Statement

Chemical Comprehensive Analysis of Avobenzone

ChemTask No. CHEM10985
MRI Project No. 110730
MRI Assignment No. 2003

This study was inspected by the Quality Assurance Unit of MRI (QAU) and the findings reported to the Study Director and Management as follows:

Phase inspected	Date inspected	Date reported
Protocol Audit	3/1/11	3/1/11
In-life Audit; Stability analysis	3/1/11	3/1/11
Protocol Amendment No. 1 Audit	2/8/12	2/10/12
Protocol Amendment No. 2 Audit	2/8/12	2/10/12
Protocol Amendment No. 3 Audit	2/8/12	2/10/12
Data Audit	2/9/12	2/10/12
Draft Final Report Audit	2/9/12	2/10/12

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	Inspection date	Management submitted date
285N laboratory complex	7/13/11	7/14/11
GC facility	7/14/11	7/15/11

MIDWEST RESEARCH INSTITUTE

[redacted]

Senior Quality Assurance Officer

Approved:

[redacted]

Director, Quality and Regulatory Systems

February 16, 2012

Good Laboratory Practice Compliance Statement

Chemical Comprehensive Analysis of Avobenzone

ChemTask No. CHEM10985
MRI Project No. 110730
MRI Assignment No. 2003

All work performed at Midwest Research Institute for this assignment was conducted in compliance with the Good Laboratory Practice regulations of the U.S. Food and Drug Administration (21 *CFR* Part 58). Elemental analysis was performed by ICON Developmental Solutions, LLC, in compliance with FDA current Good Laboratory Practices (21 *CFR* Part 58).

The raw data and report will be stored in the MRI Archives.

[redacted]
Study Director

2/16/12
Date:

RTI INTERNATIONAL
NTP Analytical Chemistry Services
3040 Cornwallis Road • PO Box 12194 • Research Triangle Park, NC 27709-2194 • USA
Telephone 919.541.6733 or 919.541.5975 • Fax 919.485.2650 • www.rti.org

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

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

[redacted]
Program Information Coordinator

HOMOSALATE

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted] 09-05-12

Task Leader — Date

Approved by:

[redacted] 09/05/12

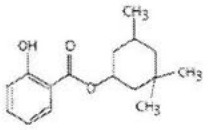
Reshan Fernando, Ph.D. — Date
Principal Investigator

Submitted to:

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

HOMOSALATE

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

STRUCTURE	MOL WT.	MOL FORMULA
	262.34	$C_{18}H_{22}O_3$

EXECUTIVE SUMMARY

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

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: Homosalate

Task Type: Chemical Reanalysis

Chem Task Number: CHEM11788

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

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Sample Preparation Inspection	05/21/12	05/21/12
Data & Report Audit	08/16/12	08/16/12

Prepared by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

Reviewed by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

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Figures

HOMOSALATE

1.0 INTRODUCTION

The objective of this work was to determine the purity and verify the identity of homosalate in support of studies being conducted at ILS. To accomplish this objective, a chemical reanalysis was performed. The identity of the chemical was confirmed by FTIR and its purity assessed by GC.

2.0 CHEMICAL ANALYSIS

An aliquot of the bulk sample of homosalate was received on March 27, 2012 for chemical reanalysis (RTI log 091410-A-14). The aliquot was stored at room temperature. A frozen reference (RTI log 091410-A-05) sample was received May 10, 2012 and was stored at freezer temperature.

3.0 CONFIRMATION OF IDENTITY - INFRARED SPECTROMETRY (IR)

3.1 IR Parameters

System	Thermo Nicolet 6700 FTIR
Software	Omnic, Ver. 7.3
Method	NaCl disks, scan 4000 - 400 cm^{-1}

3.2 Results

Bulk Sample Frequency (1/cm)	Frozen Reference Sample Frequency (1/cm)	Assignment
3150	3150	O-H stretch
2953-2869	2953-2869	C-H stretch
1672	1672	C=C, C=0 stretch
1614	1614	C=C stretch
1585	1585	C=C stretch
1089	1089	C-C, C-O stretch
757	757	C-H bend

The observed spectrum for the bulk sample matched the spectrum of the frozen reference sample, and is consistent with the structure of homosalate (as reported in the bulk chemical comprehensive task CHEM11090). Figure 1 shows the bulk and frozen reference IR spectra.

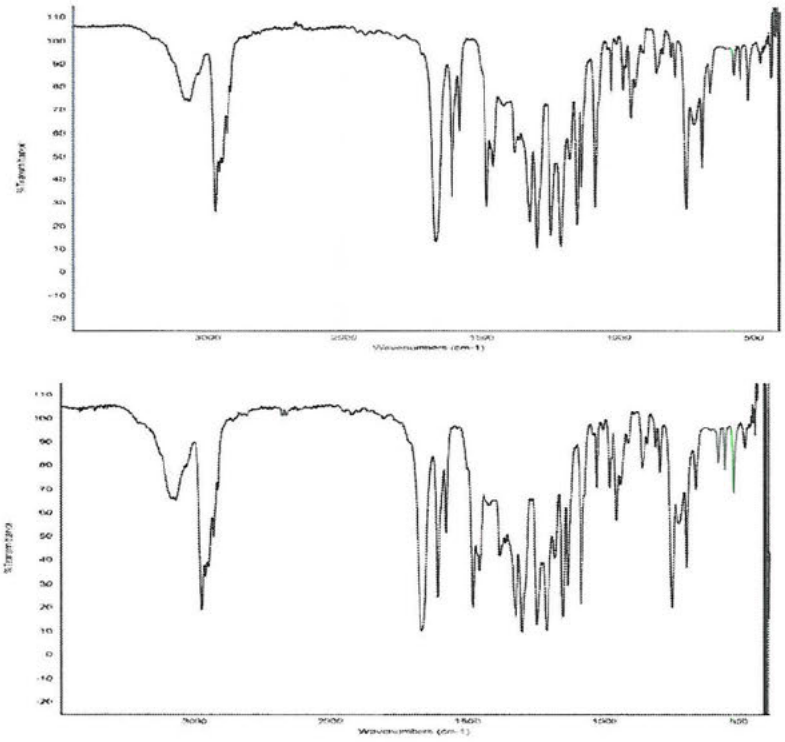


Figure 1: Infrared Spectrum of Homosalate Bulk (top spectrum) and Frozen Reference (bottom spectrum)

4.0 DETERMINATION OF PURITY - GAS CHROMATOGRAPHY

This section describes the gas chromatographic method used to estimate sample purity.

4.1 Preparation of Internal Standard (IS) Solution

A solution of IS was prepared by weighing 115.49 mg of octanophenone and transferring it into a 200-mL volumetric flask. The IS was diluted to volume with dichloromethane. The flask was mixed by inversion. The IS solution had a concentration of 0.577 mg/mL.

4.2 Bulk Sample and Frozen Reference Standard Solution Preparation

Triplicate solutions of the reference standard and bulk samples were prepared by transferring approximately 25 mg of compound to individual 25-mL volumetric flasks and diluting to volume with IS solution and mixing by inversion. An aliquot of the bulk and reference solutions were transferred to GC vials for analysis. The samples were analyzed by gas chromatography.

4.3 Analysis

GC Parameters

Instrument	Agilent 6890N GC
Data System	Empower 2; Build 2154
Column	Phenomenex ZB-5MS (30 m x 0.25 mm ID, 0.5 µm film) with 5 m pre-guard
Carrier Gas	Helium
Flow Rate	1.5 mL/min
Oven Temperature	70 °C for 1 min., ramp to 270 °C at 20 °C/min with a 7 min hold
Retention Times	Homosalate: ~11.1 min. and 11.2 min (two peaks - cis/trans isomers) Octanophenone (IS): ~9.9 min.
Injector Type and Volume	Split (20:1), 1 µL.
Injector Temperature	250 °C
Detector-Temperature	FID at 290 °C

The suitability of the system was evaluated, and the results are shown below.

Parameter	Criteria	Result	Pass/Fail
Tailing Factor, T	$0.5 \geq T \leq 2.0$	1.0	Pass
Column Efficiency, N	$\geq$ 250,000 plates	2,460,486	Pass
Precision (%RSD)	$\leq$ 5% (n=6)	0.2	Pass
Resolution	$\geq$ 40	41	Pass

4.4 Results

Calculations based on a major peak comparison technique gave the results shown in the following table. Typical chromatograms are shown in Figure 2.

RTI Log No.	Chemical	RRF[a]	Mean RRF (%RSD)	Percent Relative Purity[b]
091410-A-14	Analytical Replicate #1	1.443	1.414 (2.0)	99.3
	Analytical Replicate #2	1.412		
	Analytical Replicate #3	1.388		
091410-A-05	Reference Replicate #1	1.430	1.424 (0.69)	--
	Reference Replicate #2	1.430		
	Reference Replicate #3	1.413		

[a] RRF = Relative Response Factor; normalized to sample concentration.
[b] Relative Purity = (Mean RRF, bulk/ Mean RRF, ref.) x 100.

Based on the chromatographic results, the bulk sample had not significantly changed as compared to the frozen reference, and no significant impurities were observed.

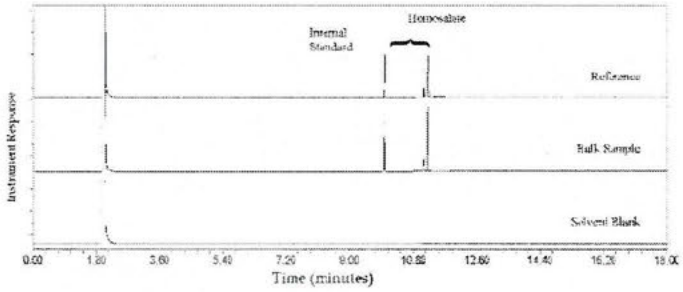


Figure 2: Example Gas Chromatograms of Homosalate Reference and Bulk Sample, and a Blank

5.0 REFERENCE

RTI International report "Homosalate, Characterization Protocols Development, (CHEM11293), January 6, 2012.

6.0 ACKNOWLEDGMENTS

Personnel contributing to this task: [redacted]

RTI INTERNATIONAL
RTP Analytical Chemistry Services
3040 Cornwallis Road • PO Box 12194 • Research Triangle Park, NC 27709-2194 • USA
Telephone 919.541.6700 or 919.541.5075 • Fax 919.485.2650 • www.rti.org

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

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

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

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:
[redacted] 09.05.12
Task Leader Date

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

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

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

CAS No.: 21245-02-3

RTI Chemical ID Code: L98

ChemTask No.: CHEM11787

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

Program Supported: TOX

Analysis Dates: May 21-22, 24, 2012

Interim Results Date: May 30, 2012

Study Lab: (Investigator): ILS [redacted]

Lot No. (Vendor): MKBF0590V (Aldrich)

Vendor Purity: 98.3% (Aldrich COA)

Receipt Date: Aug 20, 2010 (Bulk)

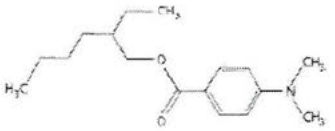
Bulk Receipt Condition: Good, room temperature

Submitter: [redacted] (RTI)

Shipping Container: NA (in-house transfer)

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

STRUCTURE



MOL. WT.
277.40

MOL. FORMULA
$C_{17}H_{27}NO_2$

EXECUTIVE SUMMARY

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

RTI INTERNATIONAL

Quality Assurance Statement

Chemical Name: 2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O)

Task Type: Chemical Reanalysis

RTI Task Number: 0212839.200.003.081

Chem Task Number: CHEM11787

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

Inspections and Audits	Inspection and Audit Date(s)	Date Inspection/Audit Report Sent to Task Leader/ Management
Sample Analysis Inspection	05/16/12	05/22/12
Data & Report Audit	08/20/12	08/20/12

Prepared by:

[redacted] 9/5/12

Quality Assurance Specialist — Date

Reviewed by:

[redacted] 9/5/12

Quality Assurance Specialist — Date

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

1.0 INTRODUCTION

The objective of this work was to determine the purity and verify the identity of 2-Ethylhexyl-p-dimethyl-aminobenzoate (padimate O) in support of studies being conducted at ILS. To accomplish this objective, a chemical reanalysis was performed. The identity of the chemical was confirmed by FTIR and its purity assessed by GC.

2.0 CHEMICAL ANALYSIS

An aliquot of the bulk sample of padimate O was received on March 27, 2012 for chemical reanalysis (RTI log 082010-B-14). The aliquot was stored at room temperature. A frozen reference (RTI log 082010-B-05) sample was received May 10, 2012 and was stored at freezer temperature.

3.0 CONFIRMATION OF IDENTITY - INFRARED SPECTROMETRY (IR)

3.1 IR Parameters

System	Thermo Nicolet 6700 FTIR
Software	Omnic, Ver. 7.3
Method	NaCl disks, scan 4000 - 400 cm^{-1}

3.2 Results

Bulk Sample Frequency (1/cm)	Frozen Reference Sample Frequency (1/cm)	Assignment
2958-2860	2958-2860	C-H Stretch
2819	2820	N-CH_3 stretch
1703	1703	C = O stretch
1609, 1527	1609, 1527	C=C Stretch
1317	1317	C-N (tertiary amine stretch)
1183	1184	C = O Stretch
1107	1107	C-O-C Stretch

The observed spectrum for the bulk sample matched the spectrum of the frozen reference sample, and is consistent with the structure of padimate O (as reported in the bulk chemical comprehensive task CHEM11089). Figure 1 shows the bulk and frozen reference IR spectra.

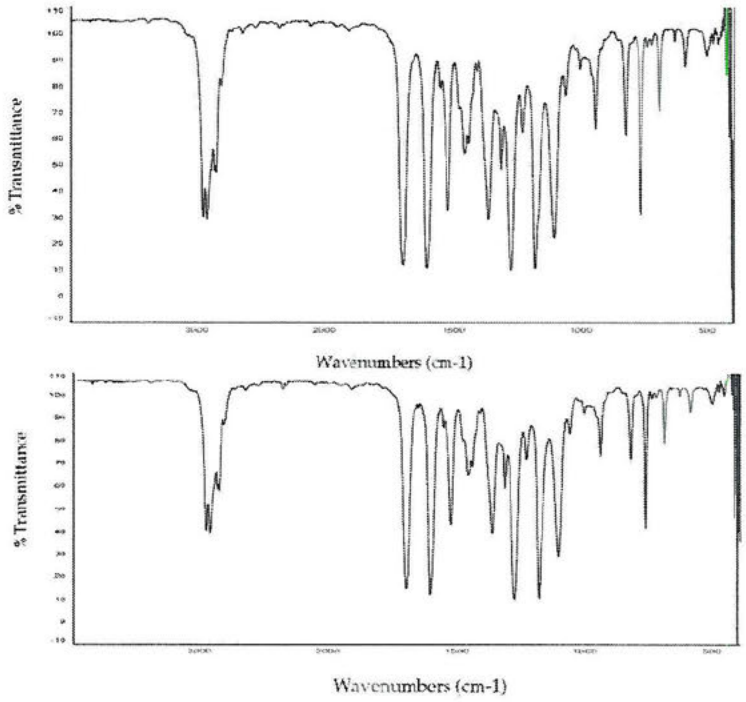


Figure 1: Infrared Spectrum of Padimate O Bulk (top spectrum) and Frozen Reference (bottom spectrum)

4.0 DETERMINATION OF PURITY - GAS CHROMATOGRAPHY

This section describes the gas chromatographic method used to estimate sample purity.

4.1 Preparation of Internal Standard (IS) Solution

A solution of IS was prepared by weighing 103.4 mg of octanophenone and transferring it into a 200-mL volumetric flask. The IS was diluted to volume with dichloromethane. The flask was mixed by inversion. The IS solution had a concentration of 0.517 mg/mL.

4.2 Bulk Sample and Frozen Reference Standard Solution Preparation

Triplicate solutions of the reference standard and bulk samples were prepared by transferring approximately 25 mg of compound to individual 25-mL volumetric flasks and diluting to volume with IS solution and mixing by inversion. An aliquot of the bulk and reference solutions were transferred to GC vials for analysis. The samples and an IS blank was analyzed by gas chromatography.

4.3 Analysis

GC Parameters

Instrument	Agilent 6890N GC
Data System	Empower 2; Build 2154
Column	Phenomenex ZB-5MS (30 m x 0.25 mm ID, 0.5 µm film) with 5 m pre-guard
Carrier Gas	Helium
Flow Rate	1.5 mL/min
Oven Temperature	70 °C for 1 min., ramp to 270°C at 20 °C/min with a 7 min hold;
Retention Times	Padimate O: ~13.6 min. ; Octanophenone (IS): ~9.9 min.
Injector Type (ratio)	Split (20:1); 1 µL
Injector Temperature	250 °C
Detector-Temperature	FID at 290 °C

The suitability of the system was evaluated, and the results are shown below.

Parameter	Criteria	Result	Pass/Fail
Tailing Factor, T	$0.5 \leq T \leq 2.0$	0.79	Pass
Column Efficiency, N	$\geq$ 250,000 plates	1,070,819	Pass
Precision (%RSD)	$\leq$ 5% (n=6)	0.6%	Pass
Resolution	$\geq$ 40	91.5	Pass

4.4 Results

Calculations based on a major peak comparison technique gave the results shown in the following table. Typical chromatograms are shown in Figure 2.

RTI Log No.	Chemical	RRF[a]	Mean RRF[a] (%RSD)	Percent Relative Purity[b]
082010-B-14	Analytical Replicate #1	1.637	1.640 (0.4)	98.1
	Analytical Replicate #2	1.647		
	Analytical Replicate #3	1.637		
082010-B-05	Reference Replicate #1	1.661	1.672 (2.1)	–
	Reference Replicate #2	1.645		
	Reference Replicate #3	1.711		

[a] RRF = Relative Response Factor; normalized to sample concentration.

[b] Relative Purity = (Mean RRF, bulk/Mean RRF, ref.) x 100.

Based on the chromatographic results, the bulk sample had not significantly changed as compared to the frozen reference, and no significant impurities were observed.

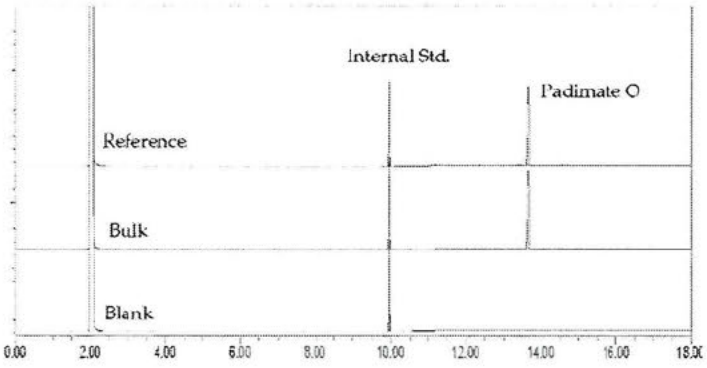


Figure 2: Example Gas Chromatograms of Padimate O Reference and Bulk Sample, and an IS Blank

5.0 REFERENCE

RTI International report "2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O), Characterization Protocols Development, (CHEM11292), January 6, 2012.

6.0 ACKNOWLEDGMENTS

Personnel contributing to this task: [redacted].

APPENDIX 14 Principal Investigator Report – OpAns, LLC

Title:

Determination of Testosterone and Estradiol in H295R Supplemented Medium Specimens from the Study Entitled, "H295R Steroidogenesis Assay"

Study Number: 9070-100794STER

Document Number: OPR-CTX-0027.01

Approved by:	**Signature:**	**Effective Date:**
Kenneth C Lewis, PhD Principal Investigator, OpAns, LLC	[redacted]	31 Jul 13

Revisions

Version	Effective Date	Description
01	31 Jul 2013	Initial document

Contents

PRINCIPAL INVESTIGATOR'S STATEMENT OF COMPLIANCE

This phase (i.e., HPLC/MS-MS measurement of testosterone and estradiol) of the study was conducted in accordance with US Environmental Protection Agency's Good Laboratory Practice Regulations as set forth in Title 40 of the CFR Part 160 (October 16, 1989).

Nothing occurred to affect adversely the quality or integrity of these experimental data.

I consider the data generated to be valid.

	Signature:	**Date:**
Kenneth C Lewis, PhD Principal Investigator OpAns, LLC.	[redacted]	31Jul13

QUALITY ASSURANCE STATEMENT

Study Number: 9070-100794STER

Report Title: Determination of Testosterone and Estradiol in H295R Supplemented Medium Specimens from the Study Entitled, "H295R Steroidogenesis Assay"

The phases, processes and documents relating to this portion of the study conducted by OpAns were audited and the results of the audits were reported to the Study Director (SD), Principal Investigator (PI) and Management. The methods and results presented in the parts of the report prepared by OpAns accurately reflect the raw data.

Associated laboratories and support functions are subject to regular audits.

Audit Phase	Audit Date(s)	Date(s) Reported to PI/SD/Management
Protocol	06 May 2013	No Findings
Run Preparation	30 May 2013	No Findings
Sample Analysis	30 May2013	No Findings
Report Audit	30 Jul 2013	31 Jul 2013*

* Report included summary of previously conducted audits resulting in no findings.

Auditor: [redacted]
Manager, Quality Assurance
OpAns, LLC

Signature: [redacted]

Date: 31 Jul 2013

Key Study Details

Sponsor:	National Institute of Environmental Health Sciences P O Box 12233 Research Triangle Park, NC 27709 (USA)
Study Monitor:	[redacted] Integrated Laboratory Systems, Inc.
Test Facility:	CeeTox, Inc 4717 Campus Drive Kalamazoo, MI 49008 (USA)
Study Director:	[redacted] CeeTox, Inc
Test Site:	OpAns, LLC 4134 South Alston Ave, Suite 101 Durham, NC 27713-1879 (USA)
Principal Investigator:	Kenneth C Lewis, PhD
Study Phase:	HPLC/MS-MS measurement of testosterone and estradiol
Analyst Involved:	[redacted]
Date of First Sample Analysis:	20 May 2013
Date of Last Sample Analysis:	03 June 2013
Primary Applications Used to Acquire Data:	Agilent MassHunter Workstation Data Acquisition for Triple Quad B.03.01 (B2065) Agilent MassHunter Quantitative Analysis for QQQ (B.04.00/Build 4.0.225.0.) Excel 2007

Location of Records:

The signed original of this report and the raw data (or exact copies thereof) generated as a result of this phase of the study will be retained at OpAns or an approved archive facility contracted by OpAns for a period up to 1 year following completion of the study (i.e., final report issue date) or until returned to the Sponsor. OpAns reserves the right to retain exact copies of these records for purposes of maintaining the capability of addressing test facility regulatory requirements.

1. OBJECTIVES AND PROCEDURES

The objective of this phase of the study was to determine the levels of testosterone and estradiol in H295R supplemented medium using HPLC/MS-MS and report the results to the Study Director.

2. ANALYTICAL METHOD

2.1. Analytical Method

Testosterone and estradiol were extracted from H295R supplemented medium by liquid liquid extraction using methyl tert-butyl ether (MTBE) after the addition of [$^2H_5$]-testosterone and [$^2H_5$]-estradiol as internal standards. Extracts were analysed by HPLC/MS-MS using positive ion multiple reaction monitoring. This method (OPM-OPP-0008, see summary in Appendix 1) was validated over the range 100 to 100000 pg/mL for testosterone and 10 to 10000 pg/mL for estradiol [OpAns Document Number OPR-OPP-0006]. The lower limit of quantification (LLOQ) was 100 pg/mL for testosterone and 10 pg/mL for estradiol using a 300 µL aliquot of H295R supplemented medium.

Freshly prepared calibration standards (n = 7) were prepared in supplemented medium for each run. QC samples spiked in supplemented medium at three concentrations (2 replicates per concentration), were included with each run. All chromatograms, from each analytical run, were reviewed to verify that the appropriate peaks had been identified and correctly integrated. Representative calibration standard chromatograms are presented in Figure 1.

2.2. Calibration Standards Acceptance Criteria

Matrix-based calibration standards were deemed acceptable if the back-calculated concentration fell within ±15%, except for LLOQ, when it fell within ±20% for at least 75% (or a minimum of six standards) of the calibration standards. Values falling outside of these limits were discarded. Results for the back-calculated calibration standards for each accepted analytical run are presented in Tables 1 and 2.

2.3. Quality Control Acceptance Criteria

Quality Control (QC) samples spiked in supplemented medium at a minimum of three concentrations (one with 3x of the LLOQ (QC 30 300)), one in the midrange (QC 800 8000), and one approaching the high end of the range (QC 8000 80000) were incorporated into each run, two samples at each concentration. The results of the QC samples provided the basis for accepting or rejecting the analytical run. At least 67% (four out of six) of the QC samples were within 30% of their respective nominal (theoretical) values.

Results of QC samples analysed within the study, along with precision and accuracy data are presented in Tables 3 and 4.

2.4. Preparation and Storage of Quality Control Samples

QC samples were prepared for testosterone and estradiol at three concentrations (300, 8000, and 80000 pg/mL for testosterone and 30, 800, and 8000 pg/mL for estradiol). The QC samples were prepared on 25 April 2013 and stored frozen at -80°C with the study samples.

3. STUDY SPECIMENS

3.1. Specimen Management

All study specimens were received in acceptable condition (dry ice (solid CO_2).) Specimens were stored at -80°C then thawed at room temperature prior to analysis.

3.2. Data Analysis

HPLC/MS-MS data were acquired using proprietary software application MassHunter Workstation Acquisition (Version B.03.01 (B2065), Agilent Technologies, Inc.). Data were processed (integrated) using the software application MassHunter Quantitative Analysis for QQQ (version B.04.00/Build 4.0.225.0. Agilent Technologies, Inc.) Calibration plots of area ratio versus testosterone and estradiol concentrations were constructed and a weighted $1/x^2$ linear regression applied to the data using MassHunter Quantitative Analysis for QQQ. Statistical calculations such as means, standard deviations, etc. were performed using Excel 2007. Sample results are presented in Table 5.

3.3. Repeat Analyses

No study samples were re-analysed during this study.

4. REFERENCES

OpAns Document Number OPR-OPP-0006.01 Study No. OPP-OPP-0003. The validation of a method for the determination of testosterone and estradiol and in H295R cell medium by HPLC/MS-MS.

TABLES

Table 1 Summary of Back-Calculated Calibration Testosterone Standards for Study 9070-100794STER

Analytical Run ID	Calibration Standard Concentration *pg/mL*							Slope	Intercept	Corr. Coeff.
	100	200	1000	5000	10000	50000	100000			
9070-100794STER Run 1	103	187	1017	4898	9454	55061	105735	0.000105	-0.000503	0.995560
	104	180	1049	4750	9825	NR	100334			
9070-100794STER Run 2	102	185	979	4847	9328	53207	104129	0.000110	-0.000444	0.996145
	104	191	1050	4735	9781	55208	99404			
9070-100794STER Run 3	109	182	1073	5033	9232	48469	103554	0.000102	0.000630	0.994808
	98	188	1008	4986	9566	56224	98806			
9070-100794STER Run 4	NR	209	1041	4734	9671	49110	104241	0.002041	0.010651	0.998317
	97	202	968	4885	9679	52197	103545			
9070-100794STER Run 5	101	205	1044	4926	9460	51015	104989	0.000110	-0.000264	0.996573
	104	173	1017	4781	9785	51763	102719			
9070-100794STER Run 6	99	213	1022	4729	10375	50137	111005	0.000109	-0.000992	0.995161
	105	172	953	4787	10004	50213	100066			
9070-100794STER Run 7	102	187	996	4949	10420	53046	107749	0.000107	-0.000697	0.996212
	101	204	901	4756	9500	53546	97212			
9070-100794STER Run 8	96	181	1037	4967	10120	53296	105901	0.000105	0.000269	0.994480
	112	182	1015	4595	9995	52161	95201			
9070-100794STER Run 9	96	187	1077	5011	9860	54614	102539	0.000104	-0.001168	0.995368
	110	182	1034	4894	9522	48621	96554			

Summary Statistics	Calibration Standard Concentration *pg/mL*							Slope	Intercept	Corr. Coeff.
	100	200	1000	5000	10000	50000	100000			
Mean	102.6814	189.54653	1015.5464	4847.9553	9754.405	52228.62889	102426.8695	0.000	0.001	0.996
Standard Deviation	4.7798	12.0389	43.9072	120.3957	334.1924	2372.4455	4104.6254	0.0006	0.0037	0.0012
Precision (%)	4.6550	6.3514	4.3235	2.4834	3.4261	4.5424	4.0074	--	--	--
Accuracy (%)	102.7	94.8	101.6	97.0	97.5	104.5	102.4	--	--	--
n	17	18	18	18	18	17	18	9	9	9

Statistics calculated from non-rounded data.
*Excluded from calculations, did not meet acceptance criteria due to analytical reason. NR due to a bad injection.

Table 2 Summary of Back-Calculated Estradiol Calibration Standards for Study 9070-100794STER

Analytical Run ID	Calibration Standard Concentration *pg/mL*							Slope	Intercept	Corr. Coeff.
	10	20	100	500	1000	5000	10000			
9070-100794STER Run 1	10.0	19.1	96.8	475.9	903.9	5424.7	10502.7	0.0019	-0.0020	0.9960
	*17.0	20.9	101.3	*411.8	*795.8	NR	10251.6			
9070-100794STER Run 2	9.7	17.8	97.9	482.4	941.9	5449.1	10917.1	0.0018	-0.0011	0.9933
	11.0	19.4	101.1	452.3	959.2	5424.5	10387.8			
9070-100794STER Run 3	10.2	16.5	103.9	512.3	911.0	4967.5	10723.1	0.0017	0.0001	0.9972
	10.1	*18.8	98.7	503.5	950.5	*5962.6	10479.3			
9070-100794STER Run 4	NR	19.9	103.5	479.2	1022.9	5282.9	10589.0	0.0042	-0.0037	0.9970
	10.3	19.0	100.1	472.3	912.9	5316.5	9775.6			
9070-100794STER Run 5	11.0	19.9	103.2	484.4	949.4	5345.4	10847.5	0.0019	-0.0014	0.9941
	9.6	17.9	94.7	461.4	954.4	5308.5	10607.7			
9070-100794STER Run 6	10.5	21.7	99.9	483.5	1025.5	5215.8	11296.6	0.0019	-0.0009	0.9942
	9.2	*15.4	90.3	456.8	961.8	4963.9	10100.5			
9070-100794STER Run 7	11.4	19.0	98.8	512.4	1063.6	5728.9	*11771.0	0.0018	-0.0007	0.9886
	9.5	17.9	86.3	468.5	931.1	5512.1	10067.6			
9070-100794STER Run 8	11.3	18.6	100.7	503.2	1029.8	5597.9	*11726.4	0.0018	0.0006	0.9932
	9.5	18.4	98.4	451.8	1000.8	5299.0	9634.8			
9070-100794STER Run 9	10.8	18.3	111.5	502.7	999.9	5719.3	11206.9	0.0018	-0.0008	0.9901
	10.1	17.6	99.3	466.3	895.7	4759.6	9551.8			

Summary Statistics	Calibration Standard Concentration *pg/mL*							Slope	Intercept	Corr. Coeff.
	10	20	100	500	1000	5000	10000			
Mean	10.270715	18.852225	99.248476	480.51494	965.54107	5332.2247	10433.727	0.002	-0.001	0.994
Standard Deviation	0.6935	1.2998	5.3815	20.2842	49.9259	265.2696	517.8038	0.0008	0.0012	0.0029
Precision (%)	6.7525	6.8948	5.4222	4.2213	5.1708	4.9748	4.9628	--	--	--
Accuracy (%)	102.7	94.3	99.2	96.1	96.6	106.6	104.3	--	--	--
n	16	16	18	17	17	16	16	9	9	9

Statistics calculated from non-rounded data.
*Excluded from calculations, did not meet acceptance criteria due to analytical reason.

Table 3 Summary of Testosterone Quality Control Data for Study 9070-100794STER

Analytical Run ID	Quality Control Sample Concentration *pg/mL*					
	300	Accuracy (%)	8000	Accuracy (%)	80000	Accuracy (%)
9070-100794STER Run 1	304	101.4	7260	90.8	83533	104.4
	317	105.7	7462	93.3	NR	-
9070-100794STER Run 2	287	95.6	6975	87.2	73778	92.2
	307	102.4	7658	95.7	75125	93.9
9070-100794STER Run 3	305	101.6	7348	91.8	79306	99.1
	288	96.1	7556	94.4	76246	95.3
9070-100794STER Run 4	313	104.2	7307	91.3	74068	92.6
	276	92.0	7295	91.2	78891	98.6
9070-100794STER Run 5	304	101.4	7517	94.0	79587	99.5
	300	100.0	7670	95.9	81110	101.4
9070-100794STER Run 6	272	90.5	6988	87.3	76436	95.5
	276	91.9	7085	88.6	71251	89.1
9070-100794STER Run 7	304	101.3	7231	90.4	84504	105.6
	286	95.3	7351	91.9	76151	95.2
9070-100794STER Run 8	310	103.4	7956	99.4	85538	106.9
	313	104.3	7923	99.0	92344	115.4
9070-100794STER Run 9	281	93.6	7616	95.2	80773	101.0
	278	92.7	7149	89.4	71527	89.4

Summary Statistics	Quality Control Sample Concentration *pg/mL*		
	300	8000	80000
Mean	295.6032574	7408.023834	78833.50992
Standard Deviation	14.9674	286.4244	5500.0757
Precision (%)	5.0633	3.8664	6.9768
Accuracy (%)	98.5	92.6	98.5
n	18	18	17

Statistics calculated from non-rounded data.
NR due to a bad injection.

Table 4 Summary of Estradiol Quality Control Data for Study 9070-100794STER

Analytical Run ID	Quality Control Sample Concentration *pg/mL*					
	30	Accuracy (%)	800	Accuracy (%)	8000	Accuracy (%)
9070-100794STER Run 1	26.8	89.2	724.6	90.6	7773.9	97.2
	30.3	100.9	718.3	89.8	NR	-
9070-100794STER Run 2	26.8	89.2	728.2	91.0	7320.1	91.5
	27.7	92.5	790.3	98.8	7634.9	95.4
9070-100794STER Run 3	27.1	90.2	774.7	96.8	7846.4	98.1
	29.8	99.5	840.2	105.0	7825.4	97.8
9070-100794STER Run 4	28.9	96.3	753.9	94.2	7809.4	97.6
	25.4	84.7	734.0	91.7	7747.8	96.8
9070-100794STER Run 5	29.2	97.5	822.2	102.8	7975.7	99.7
	27.3	91.0	804.4	100.6	8221.5	102.8
9070-100794STER Run 6	25.8	85.9	753.8	94.2	7504.5	93.8
	26.7	88.9	727.2	90.9	6897.1	86.2
9070-100794STER Run 7	29.8	99.3	792.6	99.1	8702.8	108.8
	26.5	88.4	733.2	91.6	7483.8	93.5
9070-100794STER Run 8	28.6	95.4	844.6	105.6	8638.7	108.0
	28.4	94.6	825.3	103.2	9425.7	117.8
9070-100794STER Run 9	27.8	92.8	832.2	104.0	8680.3	108.5
	27.6	92.0	736.7	92.1	6828.5	85.4

Summary Statistics	Quality Control Sample Concentration *pg/mL*		
	30	800	8000
Mean	27.80289103	774.2455271	7900.969875
Standard Deviation	1.4182	45.0809	669.9718
Precision (%)	5.1009	5.8226	8.4796
Accuracy (%)	92.7	96.8	98.8
n	18	18	17

Statistics calculated from non-rounded data.
NR due to a bad injection.

Table 5 Testosterone and Estradiol Concentrations in H295R Media Samples

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130402 Background G1	1568	NQ	1
20130402 Background G2	1529	NQ	1
20130402 Background G3	1072	NQ	1
20130402 Background G4	1570	NQ	1
20130402 Background G5	1203	NQ	1
20130402 Background G6	1786	NQ	1
20130402 Blank A1	2958	77.1	1
20130402 Blank A2	2872	77.4	1
20130402 Blank A3	1850	51.1	1
20130402 Blank A4	2737	68.6	1
20130402 Blank A5	3037	81.5	1
20130402 Blank A6	3401	69.3	1
20130402 DMSO B1	2775	66.5	1
20130402 DMSO B2	3062	70.8	1
20130402 DMSO B3	2514	66.2	1
20130402 DMSO B4	2892	73.1	1
20130402 DMSO B5	3023	67.4	1
20130402 DMSO B6	3465	64.1	1
20130402 Prochloraz 1uM E1	787	20.2	1
20130402 Prochloraz 1uM E2	1051	30.3	1
20130402 Prochloraz 1uM E3	1042	27.6	1
20130402 Prochloraz 1uM E4	973	25.3	1
20130402 Prochloraz 1uM E5	1027	26.8	1
20130402 Prochloraz 1uM E6	1171	26.4	1
20130402 Prochloraz 0.1uM F1	1528	40.0	1
20130402 Prochloraz 0.1uM F2	2290	62.2	1
20130402 Prochloraz 0.1uM F3	2258	64.5	1
20130402 Prochloraz 0.1uM F4	2259	61.8	1
20130402 Prochloraz 0.1uM F5	2366	64.0	1
20130402 Prochloraz 0.1uM F6	3342	71.9	1
20130402 Forskolin 1uM C1	4943	320.1	1
20130402 Forskolin 1uM C2	6104	453.4	1
20130402 Forskolin 1uM C3	4823	356.7	1
20130402 Forskolin 1uM C4	5554	420.5	1
20130402 Forskolin 1uM C5	6096	470.5	1
20130402 Forskolin 1uM C6	7483	484.5	1
20130402 Forskolin 10uM D1	7830	779.6	1
20130402 Forskolin 10uM D2	7268	755.7	1
20130402 Forskolin 10uM D3	6837	703.9	1
20130402 Forskolin 10uM D4	6454	696.1	1
20130402 Forskolin 10uM D5	7320	789.3	1
20130402 Forskolin 10uM D6	9338	825.9	1

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130402 DMSO E and A A1	2891	65.3	4
20130402 DMSO E and A A2	2884	76.2	4
20130402 DMSO E and A A3	3396	79.8	4
20130402 DMSO E and A A4	3012	68.4	4
20130402 DMSO E and A B5	3039	73.0	4
20130402 DMSO E and A A6	2743	69.1	4
20130402 E Conc 1 B1	3035	68.3	4
20130402 E Conc 1 B2	3049	69.3	4
20130402 E Conc 1 B3	2765	64.8	4
20130402 E Conc 1 B4	1661	41.6	4
20130402 E Conc 1 B5	1319	34.8	4
20130402 E Conc 1 B6	1836	43.4	4
20130402 E Conc 2 C1	2672	63.3	4
20130402 E Conc 2 C2	2935	71.0	4
20130402 E Conc 2 C3	1970	44.7	4
20130402 E Conc 2 C4	2482	56.2	4
20130402 E Conc 2 C5	1110	27.7	4
20130402 E Conc 2 C6	3062	70.8	4
20130402 E Conc 3 D1	1740	39.0	4
20130402 E Conc 3 D2	2955	71.6	4
20130402 E Conc 3 D3	1783	40.7	4
20130402 E Conc 3 D4	1626	40.5	4
20130402 E Conc 3 D5	1886	47.2	4
20130402 E Conc 3 D6	3046	72.3	4
20130402 E Conc 4 E1	1947	50.1	4
20130402 E Conc 4 E2	2987	77.1	4
20130402 E Conc 4 E3	1580	41.8	4
20130402 E Conc 4 E4	2745	69.3	4
20130402 E Conc 4 E5	2152	51.0	4
20130402 E Conc 4 E6	1887	48.3	4
20130402 E Conc 5 F1	2826	67.9	4
20130402 E Conc 5 F2	2294	53.1	4
20130402 E Conc 5 F3	3161	76.0	4
20130402 E Conc 5 F4	3097	76.7	4
20130402 E Conc 5 F5	2385	64.2	4
20130402 E Conc 5 F6	2540	56.7	4
20130402 E Conc 6 G1	2611	59.1	4
20130402 E Conc 6 G2	3072	72.9	4
20130402 E Conc 6 G3	2882	68.9	4
20130402 E Conc 6 G4	2873	69.0	4
20130402 E Conc 6 G5	2414	67.5	4
20130402 E Conc 6 G6	1418	30.9	4
20130402 E Conc 7 H1	2556	58.2	4
20130402 E Conc 7 H2	2816	68.4	4
20130402 E Conc 7 H3	2822	69.6	4

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130402 E Conc 7 H4	2706	67.9	4
20130402 E Conc 7 H5	2311	66.0	4
20130402 E Conc 7 H6	2257	49.9	4
20130402 DMSO E and A A7	2171	51.9	4
20130402 DMSO E and A A8	1658	43.5	4
20130402 DMSO E and A A9	2197	NR	4
20130402 DMSO E and A A10	1424	36.4	4
20130402 DMSO E and A A11	2877	77.4	4
20130402 DMSO E and A A12	2935	75.3	4
20130402 A Conc 1 B7	1688	13.1	4
20130402 A Conc 1 B8	2248	15.1	4
20130402 A Conc 1 B9	1988	13.4	4
20130402 A Conc 1 B10	1958	14.9	4
20130402 A Conc 1 B11	2147	17.1	4
20130402 A Conc 1 B12	2124	16.5	4
20130402 A Conc 2 C7	2469	60.2	4
20130402 A Conc 2 C8	1622	41.6	4
20130402 A Conc 2 C9	1884	49.0	4
20130402 A Conc 2 C10	2774	72.2	4
20130402 A Conc 2 C11	2482	61.9	4
20130402 A Conc 2 C12	3172	70.8	4
20130402 A Conc 3 D7	2405	58.1	4
20130402 A Conc 3 D8	1726	44.0	4
20130402 A Conc 3 D9	1539	43.4	4
20130402 A Conc 3 D10	1722	39.5	4
20130402 A Conc 3 D11	2906	73.6	4
20130402 A Conc 3 D12	3030	69.3	4
20130402 A Conc 4 E7	2417	57.7	4
20130402 A Conc 4 E8	1209	28.7	4
20130402 A Conc 4 E9	1690	50.3	4
20130402 A Conc 4 E10	1918	50.1	4
20130402 A Conc 4 E11	1976	45.3	4
20130402 A Conc 4 E12	3217	72.0	4
20130402 A Conc 5 F7	2290	55.0	4
20130402 A Conc 5 F8	2659	69.1	4
20130402 A Conc 5 F9	2849	76.6	4
20130402 A Conc 5 F10	1679	45.5	4
20130402 A Conc 5 F11	3065	76.8	4
20130402 A Conc 5 F12	1730	38.8	4
20130402 A Conc 6 G7	2451	61.5	4
20130402 A Conc 6 G8	2839	71.2	4
20130402 A Conc 6 G9	2825	76.6	4
20130402 A Conc 6 G10	2911	81.8	4
20130402 A Conc 6 G11	3102	74.2	4
20130402 A Conc 6 G12	2788	60.1	4

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130402 A Conc 7 H7	2499	56.0	4
20130402 A Conc 7 H8	3006	70.3	4
20130402 A Conc 7 H9	3020	73.3	4
20130402 A Conc 7 H10	3007	72.1	4
20130402 A Conc 7 H11	2925	71.4	4
20130402 A Conc 7 H12	2961	67.4	4
20130402 DMSO H and P A1	2731	73.1	5
20130402 DMSO H and P A2	3079	85.5	5
20130402 DMSO H and P A3	2862	81.0	5
20130402 DMSO H and P A4	2012	58.1	5
20130402 DMSO H and P A5	3054	78.7	5
20130402 DMSO H and P A6	2509	60.1	5
20130402 H Conc 1 B1	2749	92.7	5
20130402 H Conc 1 B2	3129	113.7	5
20130402 H Conc 1 B3	2837	96.8	5
20130402 H Conc 1 B4	2190	73.4	5
20130402 H Conc 1 B5	1811	70.8	5
20130402 H Conc 1 B6	3148	102.0	5
20130402 H Conc 2 C1	2703	77.4	5
20130402 H Conc 2 C2	3130	91.6	5
20130402 H Conc 2 C3	1935	58.7	5
20130402 H Conc 2 C4	2286	63.8	5
20130402 H Conc 2 C5	2024	59.2	5
20130402 H Conc 2 C6	2130	60.3	5
20130402 H Conc 3 D1	2974	74.1	5
20130402 H Conc 3 D2	2587	69.6	5
20130402 H Conc 3 D3	2013	53.5	5
20130402 H Conc 3 D4	2537	60.4	5
20130402 H Conc 3 D5	1847	45.5	5
20130402 H Conc 3 D6	1919	46.1	5
20130402 H Conc 4 E1	3044	77.4	5
20130402 H Conc 4 E2	2486	65.6	5
20130402 H Conc 4 E3	2297	54.7	5
20130402 H Conc 4 E4	2618	69.2	5
20130402 H Conc 4 E5	1575	43.2	5
20130402 H Conc 4 E6	2320	56.6	5
20130402 H Conc 5 F1	2807	67.2	5
20130402 H Conc 5 F2	2358	58.6	5
20130402 H Conc 5 F3	2595	62.1	5
20130402 H Conc 5 F4	3046	72.1	5
20130402 H Conc 5 F5	1668	41.3	5
20130402 H Conc 5 F6	2051	48.9	5
20130402 H Conc 6 G1	2265	55.9	5
20130402 H Conc 6 G2	2415	60.7	5
20130402 H Conc 6 G3	2843	70.4	5

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130402 H Conc 6 G4	1732	40.6	5
20130402 H Conc 6 G5	1996	46.1	5
20130402 H Conc 6 G6	2951	66.1	5
20130402 H Conc 7 H1	2114	46.5	5
20130402 H Conc 7 H2	2599	63.8	5
20130402 H Conc 7 H3	2722	66.1	5
20130402 H Conc 7 H4	2876	68.3	5
20130402 H Conc 7 H5	2524	59.6	5
20130402 H Conc 7 H6	2732	61.3	5
20130402 DMSO H and P A7	2651	65.4	5
20130402 DMSO H and P A8	2957	78.0	5
20130402 DMSO H and P A9	2915	81.4	5
20130402 DMSO H and P A10	3175	76.5	5
20130402 DMSO H and P A11	1934	49.1	5
20130402 DMSO H and P A12	2161	55.6	5
20130402 P Conc 1 B7	2908	76.1	5
20130402 P Conc 1 B8	2909	84.8	5
20130402 P Conc 1 B9	2364	70.4	5
20130402 P Conc 1 B10	3125	95.5	5
20130402 P Conc 1 B11	2827	95.5	5
20130402 P Conc 1 B12	2956	83.4	5
20130402 P Conc 2 C7	3044	86.3	5
20130402 P Conc 2 C8	2549	73.0	5
20130402 P Conc 2 C9	2722	80.5	5
20130402 P Conc 2 C10	2106	65.4	5
20130402 P Conc 2 C11	2706	82.3	5
20130402 P Conc 2 C12	3227	95.3	5
20130402 P Conc 3 D7	2763	63.9	5
20130402 P Conc 3 D8	2556	65.3	5
20130402 P Conc 3 D9	2719	68.2	5
20130402 P Conc 3 D10	2123	56.5	5
20130402 P Conc 3 D11	2153	57.6	5
20130402 P Conc 3 D12	3010	78.4	5
20130402 P Conc 4 E7	2614	62.3	5
20130402 P Conc 4 E8	2272	56.1	5
20130402 P Conc 4 E9	2456	64.6	5
20130402 P Conc 4 E10	1960	46.8	5
20130402 P Conc 4 E11	1788	49.4	5
20130402 P Conc 4 E12	2838	74.5	5
20130402 P Conc 5 F7	2888	70.2	5
20130402 P Conc 5 F8	3091	73.9	5
20130402 P Conc 5 F9	2821	72.2	5
20130402 P Conc 5 F10	2130	52.5	5
20130402 P Conc 5 F11	2114	52.5	5
20130402 P Conc 5 F12	2928	70.4	5

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130402 P Conc 6 G7	2632	61.5	5
20130402 P Conc 6 G8	2189	53.6	5
20130402 P Conc 6 G9	2631	66.5	5
20130402 P Conc 6 G10	2269	54.6	5
20130402 P Conc 6 G11	2835	67.1	5
20130402 P Conc 6 G12	2796	67.9	5
20130402 P Conc 7 H7	2764	65.2	5
20130402 P Conc 7 H8	2592	61.1	5
20130402 P Conc 7 H9	2857	71.7	5
20130402 P Conc 7 H10	2643	71.3	5
20130402 P Conc 7 H11	2671	63.5	5
20130402 P Conc 7 H12	2987	66.5	5
20130410 Background G1	1372	NQ	1
20130410 Background G2	1266	NQ	1
20130410 Background G3	1173	NQ	1
20130410 Background G4	1474	NQ	1
20130410 Background G5	1422	NQ	1
20130410 Background G6	1374	NQ	1
20130410 Blank A1	2166	41.0	1
20130410 Blank A2	2586	50.2	1
20130410 Blank A3	2745	55.9	1
20130410 Blank A4	3018	70.0	1
20130410 Blank A5	2641	50.4	1
20130410 Blank A6	2910	56.6	1
20130410 DMSO B1	2670	52.6	1
20130410 DMSO B2	2931	61.4	1
20130410 DMSO B3	2697	53.4	1
20130410 DMSO B4	2617	52.9	1
20130410 DMSO B5	2912	59.3	1
20130410 DMSO B6	2692	55.0	1
20130410 Prochloraz 1uM E1	934	21.6	1
20130410 Prochloraz 1uM E2	858	20.6	1
20130410 Prochloraz 1uM E3	877	19.7	1
20130410 Prochloraz 1uM E4	947	19.8	1
20130410 Prochloraz 1uM E5	963	22.0	1
20130410 Prochloraz 1uM E6	996	23.1	1
20130410 Prochloraz 0.1uM F1	1574	36.1	1
20130410 Prochloraz 0.1uM F2	1794	42.5	1
20130410 Prochloraz 0.1uM F3	2189	48.5	1
20130410 Prochloraz 0.1uM F4	2290	50.1	1
20130410 Prochloraz 0.1uM F5	2119	52.3	1
20130410 Prochloraz 0.1uM F6	2366	55.7	1

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130410 Forskolin 1uM C1	3356	304.9	1
20130410 Forskolin 1uM C2	4016	410.3	1
20130410 Forskolin 1uM C3	4459	482.3	1
20130410 Forskolin 1uM C4	4362	486.9	1
20130410 Forskolin 1uM C5	4073	473.0	1
20130410 Forskolin 1uM C6	4346	460.7	1
20130410 Forskolin 10uM D1	4930	702.1	1
20130410 Forskolin 10uM D2	5703	855.6	1
20130410 Forskolin 10uM D3	5787	900.5	1
20130410 Forskolin 10uM D4	5602	917.3	1
20130410 Forskolin 10uM D5	5536	939.1	1
20130410 Forskolin 10uM D6	5876	856.2	1
20130410 DMSO E and A A1	3105	62.9	6
20130410 DMSO E and A A2	2885	64.9	6
20130410 DMSO E and A A3	2646	54.9	6
20130410 DMSO E and A A4	3282	69.0	6
20130410 DMSO E and A B5	2778	57.1	6
20130410 DMSO E and A A6	2988	62.0	6
20130410 E Conc 1 B1	2494	84.8	6
20130410 E Conc 1 B2	2878	61.3	6
20130410 E Conc 1 B3	1637	35.1	6
20130410 E Conc 1 B4	1671	38.7	6
20130410 E Conc 1 B5	1281	27.9	6
20130410 E Conc 1 B6	2694	56.3	6
20130410 E Conc 2 C1	3217	71.4	6
20130410 E Conc 2 C2	1412	31.3	6
20130410 E Conc 2 C3	1535	34.7	6
20130410 E Conc 2 C4	1756	36.0	6
20130410 E Conc 2 C5	1573	31.4	6
20130410 E Conc 2 C6	1669	31.9	6
20130410 E Conc 3 D1	3566	73.0	6
20130410 E Conc 3 D2	2813	60.4	6
20130410 E Conc 3 D3	1595	33.9	6
20130410 E Conc 3 D4	1493	30.2	6
20130410 E Conc 3 D5	1377	27.8	6
20130410 E Conc 3 D6	2321	46.5	6
20130410 E Conc 4 E1	3391	68.1	6
20130410 E Conc 4 E2	1494	33.2	6
20130410 E Conc 4 E3	1368	28.2	6
20130410 E Conc 4 E4	1333	26.1	6
20130410 E Conc 4 E5	1221	24.5	6

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130410 E Conc 4 E6	1544	32.5	6
20130410 E Conc 5 F1	3486	66.6	6
20130410 E Conc 5 F2	1766	36.5	6
20130410 E Conc 5 F3	1490	31.2	6
20130410 E Conc 5 F4	1955	38.4	6
20130410 E Conc 5 F5	1044	21.3	6
20130410 E Conc 5 F6	1541	30.2	6
20130410 E Conc 6 G1	3454	67.1	6
20130410 E Conc 6 G2	1619	35.7	6
20130410 E Conc 6 G3	3063	59.3	6
20130410 E Conc 6 G4	2086	45.3	6
20130410 E Conc 6 G5	2633	57.4	6
20130410 E Conc 6 G6	2746	52.7	6
20130410 E Conc 7 H1	2918	57.8	6
20130410 E Conc 7 H2	2822	59.1	6
20130410 E Conc 7 H3	3228	64.4	6
20130410 E Conc 7 H4	2684	56.2	6
20130410 E Conc 7 H5	3126	59.8	6
20130410 E Conc 7 H6	3221	57.2	6
20130410 DMSO E and A A7	3399	65.7	6
20130410 DMSO E and A A8	3323	66.0	6
20130410 DMSO E and A A9	2240	47.2	6
20130410 DMSO E and A A10	3084	64.8	6
20130410 DMSO E and A A11	3148	64.3	6
20130410 DMSO E and A A12	3534	63.2	6
20130410 A Conc 1 B7	1080	13.2	6
20130410 A Conc 1 B8	783	11.1	6
20130410 A Conc 1 B9	808	10.6	6
20130410 A Conc 1 B10	1523	21.6	6
20130410 A Conc 1 B11	1258	19.0	6
20130410 A Conc 1 B12	1287	18.9	6
20130410 A Conc 2 C7	2211	51.7	6
20130410 A Conc 2 C8	1497	33.8	6
20130410 A Conc 2 C9	1786	37.1	6
20130410 A Conc 2 C10	1548	32.5	6
20130410 A Conc 2 C11	2599	55.8	6
20130410 A Conc 2 C12	2982	59.6	6
20130410 A Conc 3 D7	1769	32.2	6
20130410 A Conc 3 D8	1715	32.6	6
20130410 A Conc 3 D9	1690	31.6	6
20130410 A Conc 3 D10	1650	29.6	6

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130410 A Conc 3 D11	1959	36.1	6
20130410 A Conc 3 D12	3135	59.5	6
20130410 A Conc 4 E7	2013	38.3	6
20130410 A Conc 4 E8	1573	31.2	6
20130410 A Conc 4 E9	1869	35.9	6
20130410 A Conc 4 E10	1829	35.2	6
20130410 A Conc 4 E11	2731	49.3	6
20130410 A Conc 4 E12	3013	52.5	6
20130410 A Conc 5 F7	1732	35.4	6
20130410 A Conc 5 F8	1844	36.5	6
20130410 A Conc 5 F9	1979	38.2	6
20130410 A Conc 5 F10	2961	56.0	6
20130410 A Conc 5 F11	2442	46.8	6
20130410 A Conc 5 F12	2654	44.9	6
20130410 A Conc 6 G7	3223	64.0	6
20130410 A Conc 6 G8	3147	60.0	6
20130410 A Conc 6 G9	2615	46.5	6
20130410 A Conc 6 G10	2361	48.3	6
20130410 A Conc 6 G11	2935	55.2	6
20130410 A Conc 6 G12	2840	52.6	6
20130410 A Conc 7 H7	3435	67.9	6
20130410 A Conc 7 H8	2711	54.3	6
20130410 A Conc 7 H9	2733	45.5	6
20130410 A Conc 7 H10	2704	50.9	6
20130410 A Conc 7 H11	2593	48.8	6
20130410 A Conc 7 H12	2826	49.5	6
20130410 DMSO H and P A1	2843	64.7	7
20130410 DMSO H and P A2	2581	51.3	7
20130410 DMSO H and P A3	3101	59.5	7
20130410 DMSO H and P A4	2905	62.9	7
20130410 DMSO H and P A5	2916	64.1	7
20130410 DMSO H and P A6	3858	70.2	7
20130410 H Conc 1 B1	2858	86.1	7
20130410 H Conc 1 B2	2472	80.8	7
20130410 H Conc 1 B3	2138	67.9	7
20130410 H Conc 1 B4	1972	59.1	7
20130410 H Conc 1 B5	2015	71.6	7
20130410 H Conc 1 B6	2451	84.8	7
20130410 H Conc 2 C1	3099	77.6	7
20130410 H Conc 2 C2	2447	63.1	7
20130410 H Conc 2 C3	2429	63.2	7

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130410 H Conc 2 C4	2642	65.2	7
20130410 H Conc 2 C5	2173	55.1	7
20130410 H Conc 2 C6	2386	55.9	7
20130410 H Conc 3 D1	3092	65.9	7
20130410 H Conc 3 D2	2826	65.0	7
20130410 H Conc 3 D3	1800	41.4	7
20130410 H Conc 3 D4	2047	43.0	7
20130410 H Conc 3 D5	2369	49.6	7
20130410 H Conc 3 D6	2303	45.7	7
20130410 H Conc 4 E1	3110	70.8	7
20130410 H Conc 4 E2	3093	66.4	7
20130410 H Conc 4 E3	2669	51.9	7
20130410 H Conc 4 E4	2181	44.5	7
20130410 H Conc 4 E5	2642	52.7	7
20130410 H Conc 4 E6	2396	52.2	7
20130410 H Conc 5 F1	3278	64.1	7
20130410 H Conc 5 F2	2266	48.0	7
20130410 H Conc 5 F3	1873	38.4	7
20130410 H Conc 5 F4	2063	40.5	7
20130410 H Conc 5 F5	2059	37.2	7
20130410 H Conc 5 F6	2444	47.7	7
20130410 H Conc 6 G1	3122	58.6	7
20130410 H Conc 6 G2	3186	69.2	7
20130410 H Conc 6 G3	3486	67.4	7
20130410 H Conc 6 G4	2361	46.5	7
20130410 H Conc 6 G5	2609	47.9	7
20130410 H Conc 6 G6	3031	56.3	7
20130410 H Conc 7 H1	3156	53.7	7
20130410 H Conc 7 H2	3107	60.8	7
20130410 H Conc 7 H3	3149	63.4	7
20130410 H Conc 7 H4	3239	67.7	7
20130410 H Conc 7 H5	3177	62.7	7
20130410 H Conc 7 H6	2365	43.1	7
20130410 DMSO H and P A7	2915	59.1	7
20130410 DMSO H and P A8	3112	68.8	7
20130410 DMSO H and P A9	3144	67.1	7
20130410 DMSO H and P A10	2897	59.5	7
20130410 DMSO H and P A11	2696	53.8	7
20130410 DMSO H and P A12	3186	61.5	7
20130410 P Conc 1 B7	3238	85.1	7
20130410 P Conc 1 B8	2351	67.1	7

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130410 P Conc 1 B9	2595	65.5	7
20130410 P Conc 1 B10	3320	82.9	7
20130410 P Conc 1 B11	3444	83.6	7
20130410 P Conc 1 B12	2502	56.3	7
20130410 P Conc 2 C7	3196	80.1	7
20130410 P Conc 2 C8	2920	73.3	7
20130410 P Conc 2 C9	2396	60.7	7
20130410 P Conc 2 C10	2295	56.1	7
20130410 P Conc 2 C11	2711	63.2	7
20130410 P Conc 2 C12	3145	71.9	7
20130410 P Conc 3 D7	2015	40.0	7
20130410 P Conc 3 D8	1833	39.4	7
20130410 P Conc 3 D9	2233	45.8	7
20130410 P Conc 3 D10	1825	37.1	7
20130410 P Conc 3 D11	2988	61.9	7
20130410 P Conc 3 D12	3393	58.3	7
20130410 P Conc 4 E7	2461	48.1	7
20130410 P Conc 4 E8	2074	44.7	7
20130410 P Conc 4 E9	2047	37.0	7
20130410 P Conc 4 E10	2044	41.6	7
20130410 P Conc 4 E11	2953	56.5	7
20130410 P Conc 4 E12	2046	35.2	7
20130410 P Conc 5 F7	2748	56.4	7
20130410 P Conc 5 F8	2070	44.0	7
20130410 P Conc 5 F9	2837	56.0	7
20130410 P Conc 5 F10	2228	43.5	7
20130410 P Conc 5 F11	3024	59.3	7
20130410 P Conc 5 F12	2564	47.1	7
20130410 P Conc 6 G7	2912	58.4	7
20130410 P Conc 6 G8	2850	59.8	7
20130410 P Conc 6 G9	3026	60.6	7
20130410 P Conc 6 G10	2893	54.2	7
20130410 P Conc 6 G11	3009	54.3	7
20130410 P Conc 6 G12	3129	59.8	7
20130410 P Conc 7 H7	2899	49.9	7
20130410 P Conc 7 H8	3000	58.1	7
20130410 P Conc 7 H9	2771	59.7	7
20130410 P Conc 7 H10	2731	54.5	7
20130410 P Conc 7 H11	2991	56.5	7
20130410 P Conc 7 H12	2968	55.3	7
20130416 Background G1	1558	15.5	2

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130416 Background G2	1480	12.4	2
20130416 Background G3	1450	10.4	2
20130416 Background G4	1457	10.2	2
20130416 Background G5	1510	NQ	2
20130416 Background G6	1625	10.5	2
20130416 Blank A1	3929	66.6	2
20130416 Blank A2	3371	69.5	2
20130416 Blank A3	3344	62.5	2
20130416 Blank A4	2844	51.8	2
20130416 Blank A5	2747	51.8	2
20130416 Blank A6	3117	55.6	2
20130416 DMSO B1	3163	57.4	2
20130416 DMSO B2	3680	63.1	2
20130416 DMSO B3	3284	57.5	2
20130416 DMSO B4	3359	55.5	2
20130416 DMSO B5	2118	37.5	2
20130416 DMSO B6	3614	60.9	2
20130416 Prochloraz 1uM E1	1406	32.5	2
20130416 Prochloraz 1uM E2	1176	29.3	2
20130416 Prochloraz 1uM E3	1197	29.5	2
20130416 Prochloraz 1uM E4	1055	26.2	2
20130416 Prochloraz 1uM E5	1049	24.2	2
20130416 Prochloraz 1uM E6	1182	28.8	2
20130416 Prochloraz 0.1uM F1	3141	64.5	2
20130416 Prochloraz 0.1uM F2	2851	58.1	2
20130416 Prochloraz 0.1uM F3	2463	51.5	2
20130416 Prochloraz 0.1uM F4	2667	60.0	2
20130416 Prochloraz 0.1uM F5	2697	54.1	2
20130416 Prochloraz 0.1uM F6	2993	61.5	2
20130416 Forskolin 1uM C1	4988	566.1	2
20130416 Forskolin 1uM C2	5508	724.3	2
20130416 Forskolin 1uM C3	5490	714.0	2
20130416 Forskolin 1uM C4	5449	723.0	2
20130416 Forskolin 1uM C5	4899	642.3	2
20130416 Forskolin 1uM C6	4497	529.0	2
20130416 Forskolin 10uM D1	7251	1241.3	2
20130416 Forskolin 10uM D2	4119	794.8	2
20130416 Forskolin 10uM D3	6216	1279.7	2
20130416 Forskolin 10uM D4	6250	1257.5	2
20130416 Forskolin 10uM D5	6040	1206.6	2
20130416 Forskolin 10uM D6	6925	1276.2	2

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130416 DMSO E and A A1	3631	62.6	8
20130416 DMSO E and A A2	3121	55.0	8
20130416 DMSO E and A A3	2343	38.9	8
20130416 DMSO E and A A4	2275	37.0	8
20130416 DMSO E and A B5	3634	61.1	8
20130416 DMSO E and A A6	2256	34.7	8
20130416 E Conc 1 B1	3768	65.0	8
20130416 E Conc 1 B2	3130	55.2	8
20130416 E Conc 1 B3	2228	34.4	8
20130416 E Conc 1 B4	2116	35.9	8
20130416 E Conc 1 B5	2488	40.2	8
20130416 E Conc 1 B6	2264	33.7	8
20130416 E Conc 2 C1	3394	56.4	8
20130416 E Conc 2 C2	1825	31.0	8
20130416 E Conc 2 C3	2210	35.7	8
20130416 E Conc 2 C4	2582	41.5	8
20130416 E Conc 2 C5	1821	28.4	8
20130416 E Conc 2 C6	2696	38.3	8
20130416 E Conc 3 D1	3637	57.2	8
20130416 E Conc 3 D2	3112	47.9	8
20130416 E Conc 3 D3	2218	33.3	8
20130416 E Conc 3 D4	2559	40.1	8
20130416 E Conc 3 D5	2185	33.1	8
20130416 E Conc 3 D6	2736	38.8	8
20130416 E Conc 4 E1	2984	45.9	8
20130416 E Conc 4 E2	2876	45.9	8
20130416 E Conc 4 E3	1899	29.7	8
20130416 E Conc 4 E4	2162	35.7	8
20130416 E Conc 4 E5	2169	33.2	8
20130416 E Conc 4 E6	2286	33.2	8
20130416 E Conc 5 F1	3154	47.0	8
20130416 E Conc 5 F2	3549	58.4	8
20130416 E Conc 5 F3	3095	49.6	8
20130416 E Conc 5 F4	3052	46.4	8
20130416 E Conc 5 F5	2358	35.2	8
20130416 E Conc 5 F6	1963	30.2	8
20130416 E Conc 6 G1	3823	56.7	8
20130416 E Conc 6 G2	3266	57.0	8
20130416 E Conc 6 G3	3253	53.3	8
20130416 E Conc 6 G4	2111	30.1	8
20130416 E Conc 6 G5	3395	51.6	8

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130416 E Conc 6 G6	1865	27.8	8
20130416 E Conc 7 H1	3271	49.5	8
20130416 E Conc 7 H2	3308	54.0	8
20130416 E Conc 7 H3	3578	55.4	8
20130416 E Conc 7 H4	3260	47.7	8
20130416 E Conc 7 H5	3655	51.8	8
20130416 E Conc 7 H6	3716	54.0	8
20130416 DMSO E and A A7	3217	62.7	8
20130416 DMSO E and A A8	3279	63.7	8
20130416 DMSO E and A A9	2221	40.6	8
20130416 DMSO E and A A10	3362	59.1	8
20130416 DMSO E and A A11	3497	63.5	8
20130416 DMSO E and A A12	3735	60.3	8
20130416 A Conc 1 B7	1201	15.1	8
20130416 A Conc 1 B8	1298	14.2	8
20130416 A Conc 1 B9	1382	15.6	8
20130416 A Conc 1 B10	1205	14.9	8
20130416 A Conc 1 B11	2146	23.6	8
20130416 A Conc 1 B12	1763	18.5	8
20130416 A Conc 2 C7	2188	37.6	8
20130416 A Conc 2 C8	2188	38.8	8
20130416 A Conc 2 C9	2383	42.0	8
20130416 A Conc 2 C10	2310	41.2	8
20130416 A Conc 2 C11	2809	50.9	8
20130416 A Conc 2 C12	3357	57.8	8
20130416 A Conc 3 D7	2187	36.4	8
20130416 A Conc 3 D8	2318	37.9	8
20130416 A Conc 3 D9	2115	36.6	8
20130416 A Conc 3 D10	2180	35.7	8
20130416 A Conc 3 D11	1979	34.9	8
20130416 A Conc 3 D12	3570	58.6	8
20130416 A Conc 4 E7	2545	42.4	8
20130416 A Conc 4 E8	2327	38.2	8
20130416 A Conc 4 E9	1955	31.9	8
20130416 A Conc 4 E10	2660	44.0	8
20130416 A Conc 4 E11	3246	54.0	8
20130416 A Conc 4 E12	2493	38.1	8
20130416 A Conc 5 F7	2329	36.0	8
20130416 A Conc 5 F8	2608	42.6	8
20130416 A Conc 5 F9	3260	53.6	8
20130416 A Conc 5 F10	2511	40.8	8

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130416 A Conc 5 F11	3216	54.9	8
20130416 A Conc 5 F12	3339	52.8	8
20130416 A Conc 6 G7	3753	59.8	8
20130416 A Conc 6 G8	3587	59.4	8
20130416 A Conc 6 G9	1750	26.5	8
20130416 A Conc 6 G10	3215	55.3	8
20130416 A Conc 6 G11	3058	50.5	8
20130416 A Conc 6 G12	3351	60.2	8
20130416 A Conc 7 H7	3984	68.0	8
20130416 A Conc 7 H8	3517	59.9	8
20130416 A Conc 7 H9	3529	59.1	8
20130416 A Conc 7 H10	3602	58.5	8
20130416 A Conc 7 H11	3933	60.3	8
20130416 A Conc 7 H12	3589	56.3	8
20130416 DMSO H and P A1	3096	54.1	9
20130416 DMSO H and P A2	3118	52.0	9
20130416 DMSO H and P A3	1867	32.7	9
20130416 DMSO H and P A4	2895	51.2	9
20130416 DMSO H and P A5	2289	39.0	9
20130416 DMSO H and P A6	2930	47.3	9
20130416 H Conc 1 B1	3459	84.9	9
20130416 H Conc 1 B2	2541	71.9	9
20130416 H Conc 1 B3	1913	47.3	9
20130416 H Conc 1 B4	2540	64.7	9
20130416 H Conc 1 B5	2137	59.9	9
20130416 H Conc 1 B6	2891	78.4	9
20130416 H Conc 2 C1	2510	49.4	9
20130416 H Conc 2 C2	1643	36.4	9
20130416 H Conc 2 C3	2272	42.8	9
20130416 H Conc 2 C4	2147	41.7	9
20130416 H Conc 2 C5	2119	40.1	9
20130416 H Conc 2 C6	2422	43.2	9
20130416 H Conc 3 D1	3448	54.0	9
20130416 H Conc 3 D2	2057	35.1	9
20130416 H Conc 3 D3	1894	32.8	9
20130416 H Conc 3 D4	2284	37.1	9
20130416 H Conc 3 D5	2671	44.1	9
20130416 H Conc 3 D6	2014	32.2	9
20130416 H Conc 4 E1	3460	55.4	9
20130416 H Conc 4 E2	3115	48.4	9
20130416 H Conc 4 E3	2020	31.3	9

Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130416 H Conc 4 E4	1745	29.9	9
20130416 H Conc 4 E5	2605	41.3	9
20130416 H Conc 4 E6	2398	37.4	9
20130416 H Conc 5 F1	3554	58.7	9
20130416 H Conc 5 F2	3516	55.0	9
20130416 H Conc 5 F3	1902	29.1	9
20130416 H Conc 5 F4	1622	27.9	9
20130416 H Conc 5 F5	1742	28.2	9
20130416 H Conc 5 F6	1581	24.3	9
20130416 H Conc 6 G1	3613	53.1	9
20130416 H Conc 6 G2	3214	53.4	9
20130416 H Conc 6 G3	3056	48.5	9
20130416 H Conc 6 G4	3020	48.1	9
20130416 H Conc 6 G5	1914	30.8	9
20130416 H Conc 6 G6	3425	53.7	9
20130416 H Conc 7 H1	3735	55.2	9
20130416 H Conc 7 H2	3276	54.1	9
20130416 H Conc 7 H3	3702	55.6	9
20130416 H Conc 7 H4	2316	36.5	9
20130416 H Conc 7 H5	3519	54.3	9
20130416 H Conc 7 H6	3548	55.6	9
20130416 DMSO H and P A7	3936	NR	9
20130416 DMSO H and P A8	2016	36.9	9
20130416 DMSO H and P A9	1983	33.5	9
20130416 DMSO H and P A10	3144	57.4	9
20130416 DMSO H and P A11	2076	33.7	9
20130416 DMSO H and P A12	2169	34.1	9
20130416 P Conc 1 B7	2824	68.9	9
20130416 P Conc 1 B8	2445	61.4	9
20130416 P Conc 1 B9	2027	48.2	9
20130416 P Conc 1 B10	2974	75.7	9
20130416 P Conc 1 B11	4031	93.1	9
20130416 P Conc 1 B12	3836	91.0	9
20130416 P Conc 2 C7	2712	51.6	9
20130416 P Conc 2 C8	2570	51.8	9
20130416 P Conc 2 C9	2115	40.0	9
20130416 P Conc 2 C10	1846	35.6	9
20130416 P Conc 2 C11	1964	45.3	9
20130416 P Conc 2 C12	4091	76.3	9
20130416 P Conc 3 D7	2396	41.7	9
20130416 P Conc 3 D8	1967	34.3	9

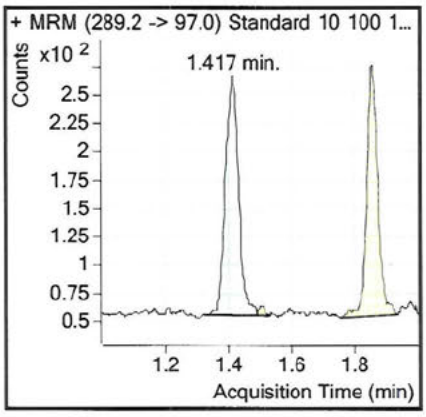
Sample Name	Testosterone Conc. pg/mL	Estradiol Conc. pg/mL	Analytical Run Number
20130416 P Conc 3 D9	2032	36.3	9
20130416 P Conc 3 D10	2141	36.7	9
20130416 P Conc 3 D11	3157	53.8	9
20130416 P Conc 3 D12	2309	35.6	9
20130416 P Conc 4 E7	2110	33.5	9
20130416 P Conc 4 E8	1654	28.9	9
20130416 P Conc 4 E9	1656	28.5	9
20130416 P Conc 4 E10	2106	35.6	9
20130416 P Conc 4 E11	2518	39.3	9
20130416 P Conc 4 E12	3045	45.3	9
20130416 P Conc 5 F7	3258	51.0	9
20130416 P Conc 5 F8	1798	30.2	9
20130416 P Conc 5 F9	1928	32.8	9
20130416 P Conc 5 F10	2892	50.1	9
20130416 P Conc 5 F11	2015	29.5	9
20130416 P Conc 5 F12	3538	56.4	9
20130416 P Conc 6 G7	3647	53.8	9
20130416 P Conc 6 G8	2491	43.4	9
20130416 P Conc 6 G9	1746	26.9	9
20130416 P Conc 6 G10	3205	51.8	9
20130416 P Conc 6 G11	3142	48.7	9
20130416 P Conc 6 G12	2930	45.4	9
20130416 P Conc 7 H7	3841	56.4	9
20130416 P Conc 7 H8	3487	53.8	9
20130416 P Conc 7 H9	3391	51.7	9
20130416 P Conc 7 H10	2063	32.6	9
20130416 P Conc 7 H11	3494	52.8	9
20130416 P Conc 7 H12	3537	52.6	9

NR=Not Reportable
NQ=Not Quantifiable

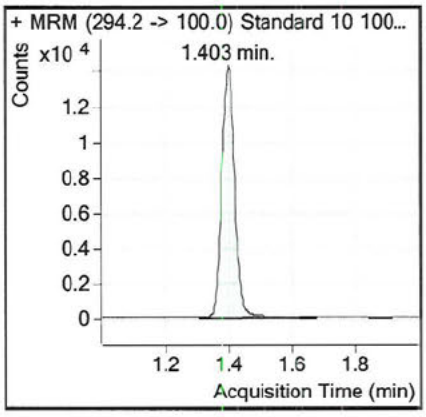
FIGURES

Figure 1 **Representative Chromatograms: Standards at LLOQ - 10 pg/mL (Estradiol) 100 pg/mL (Testosterone)**

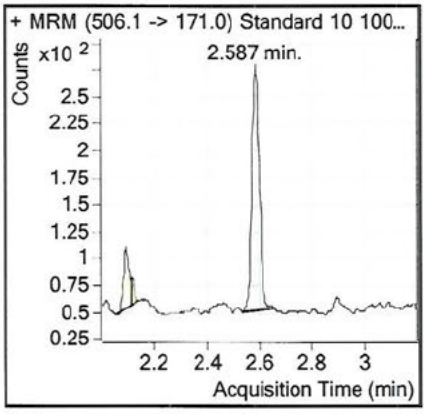
Testosterone



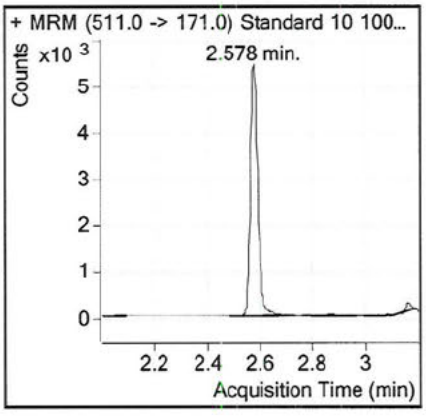
$[^2H_5]$-Testosterone



Estradiol



$[^2H_5]$-Estradiol



APPENDICES

Appendix 1 Summary of Analytical Method OPM-OPP-0008

Analytes:

Analytes	Testosterone (free alcohol)
	Estradiol (free alcohol)
Internal Standards (I.S.)	d^5-Testosterone d^5-Estradiol

Compounds are to be accurately weighed and corrected for purity and salt as necessary.

Matrices:

H295R supplemented medium

Control H295R supplemented medium is to be centrifuged for approximately 5 minutes at 4000 g prior to use if necessary.

Instrumentation Requirements:

HPLC/MS-MS

Reverse phase C18 gradient elution with electrospray positive ionization and MS/MS detection

Preparation of Calibration Standards

Calibration standards are to be prepared as follows and thoroughly mixed.

Standard Concentration (Estradiol/Testosterone pg/mL)	Volume of Working Solution (µL)							Total Volume in Control Matrix (mL)
	STDWS-200	STDWS-100	STDWS-20	STDWS-10	STDWS-2	STDWS-0.4	STDWS-0.2	
10/100	-	-	-	-	-	-	50	1
20/200	-	-	-	-	-	50	-	1
100/1000	-	-	-	-	50	-	-	1
500/5000	-	-	-	50	-	-	-	1

Standard Concentration (Estradiol/Testosterone pg/mL)	Volume of Working Solution (µL)							Total Volume in Control Matrix (mL)
	STDWS-200	STDWS-100	STDWS-20	STDWS-10	STDWS-2	STDWS-0.4	STDWS-0.2	
1000/10000	-	-	50	-	-	-	-	1
5000/50000	-	50	-	-	-	-	-	1
10000/100000	50	-	-	-	-	-	-	1

Preparation of Quality Control Samples

Quality controls (QC) are to be prepared as follows and thoroughly mixed. .

QC Concentration (Estradiol/Testosterone pg/mL)	Volume of Spiking Solution (µL)					Total Volume in Control Matrix (mL)
	QCWS-400	QCWS -200	QCWS -20	QCWS -2	QCWS -0.2	
10/100	-	-	-	-	250	5
30/300	-	-	-	375	-	25
800/8000	-	-	1000	-	-	25
8000/80000	-	1000	-	-	-	25
20000/200000	250	-	-	-	-	5

The total volumes prepared may be scaled up or down as required.

Sample Preparation:

Extraction Procedure

Aliquot sample into tube or well, and add internal standard working solution. Seal and mix for approximately 1 minute. Perform liquid/liquid extraction on samples and centrifuge for approximately 5 minutes at 4000 rpm. Transfer aliquot for analysis and evaporate to dryness. Add derivatization solution and mix. Centrifuge for approximately 1 minute at approximately 4000 rpm and inject for HPLC/MS-MS analysis.

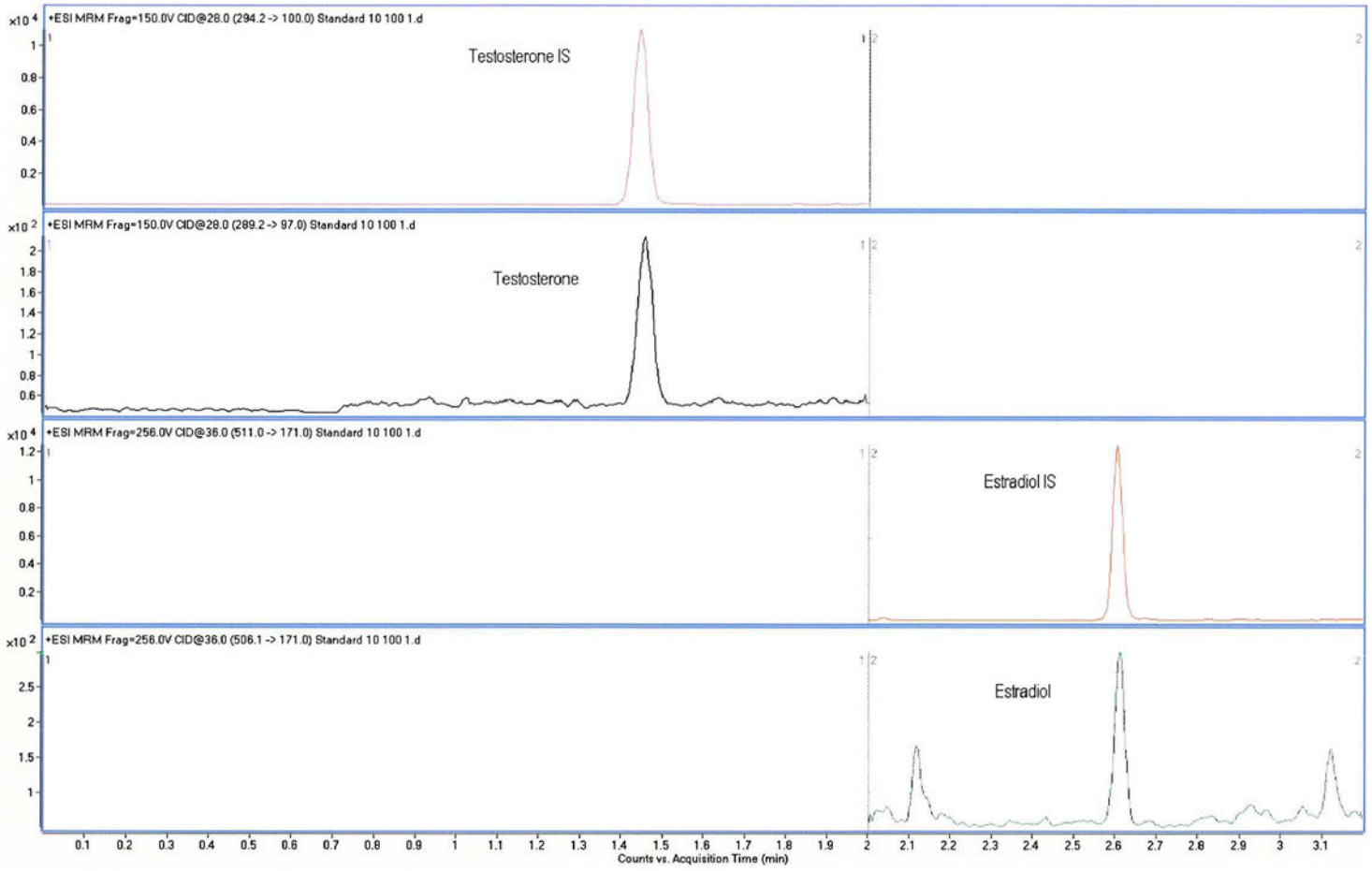
Data Analysis:

Regression Model

Use peak area ratios with $1/x^2$ weighted linear regression for all components.

Representative Chromatograms:

Example chromatogram of testosterone, estradiol, and their internal standards in H295R Supplemented Medium at approximate concentrations of 100 and 10 pg/mL, respectively:



APPENDIX 15 Study Protocol and Protocol Amendments

4717 Campus Drive, Kalamazoo, MI 49008 (269) 353-5555 (office) www.ceetox.com

CeeTox
In vitro models to predict toxicity

FINAL PROTOCOL

H295R Steroidogenesis Assay

Data Requirements: *OPPTS 890.1550*

Study Number:
9070-100794STER

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

Test Facility:
CeeTox
4717 Campus Drive
Kalamazoo, MI 49008

TEST PROTOCOL

TO BE COMPLETED BY THE STUDY SPONSOR:
Study Sponsor: NIEHS/NTP ([redacted], Chief Toxicology Branch)
Address: P.O. Box 12233
Research Triangle Park, NC **Phone:** [redacted]
Study Monitor: [redacted] **E-mail:** [redacted] **Phone:** [redacted]
CoStudy Monitor: N/A **Phone:** N/A
Sponsor Protocol/Project No: N/A
Test Substance Name(s): 2-Phenyl-5-benzimidazolesulfonic Acid (Ensulizole)
Purity: 99.6%
Batch or Lot#: 05117JE
Test Substance Name(s): Butyl-methoxydibenzoylmethane (Avobenzone)
Purity: 98.5%
Batch or Lot#: L802809
Test Substance Name(s): 3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)
Purity: 99.3%
Batch or Lot#: YT0976
Test Substance Name(s): 2-Ethylhexyl-P-Dimethyl-Aminobenzoate (Padimate-O)
Purity: 98.1%
Batch or Lot#: MKBF0590V
***Proposed Experimental Start Date:** March 15, 2013 (date subject to change; actual experimental start date to be provided in final report)
***Proposed Experimental Termination Date:** April 15, 2013 (date subject to change; actual experimental termination date to be provided in final report)

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

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

[redacted]
National Toxicology Program (NTP) Investigator
Telephone No.: [redacted]
Facsimile No.:
E-mail:

Study Monitor
[redacted]
Integrated Laboratory Systems, Inc.
Telephone No.: [redacted]
Facsimile No.:
E-mail:

Project Identification
ILS Project No.: N135
Study No.: 007
Human and Health Science Number: HHSN273200900005C
NIEHS contract number: N01ES00005

Table of Contents

Signatures

[redacted]

3/26/13
Date

Chief, Toxicology Branch
National Toxicology Program, NIEHS

[redacted]

3/25/13
Date

Contract Office Technical Representative
National Toxicology Program, NIEHS

[redacted]

19March2013
Date

Integrated Laboratory Systems, Inc.
Study Monitor

[redacted]

28 March 2013
Date

Study Director

1. Title of Study

H295R Steroidogenesis Assay

2. Purpose of Study

The purpose of this H295R Steroidogenesis Assay is to evaluate the potential of four test substances to affect the steroidogenic pathway after the gonadotropin hormone receptors (FSHR and LHR). The endpoints for the assay are testosterone and estradiol/estrone. The steroidogenic assay is not intended to identify substances that affect steroidogenesis due to effects on the hypothalamus or pituitary gland.

3. Compliance Statement

This study will be conducted in compliance with the U.S. Environmental Protection Agency Good Laboratory Practice regulations Title 40, Part 160 with the exception of section 160.113. Dose concentrations of test substance and control substances will not be verified using analytical methods.

4. Quality Assurance

This study will be subjected to periodic inspections and the final draft report will be reviewed by the Quality Assurance Unit of CeeTox in accordance with CeeTox standard operating procedures (SOP).

5. Regulatory Citations and Guidelines

OPPTS 890.1550: Steroidogenesis (Human Cell Line - H295R). 2009.

6. Test Facility and Performing Laboratory

CeeTox, Inc.
4717 Campus Drive
Kalamazoo, MI 49008
USA

Performing Laboratory for HPLC/MS/MS
OpAns LLC
4134 South Alston, Suite 101
Durham, NC 27713
Phone 919-323-4299
Principal Investigator: Kenneth Lewis, Ph.D.
Phase Performed: HPLC/MS/MS Analysis of H295R Media Samples for Testosterone and Estradiol

7. Experimental Design

This steroidogenesis assay is to be used in conjunction with other guidelines in the OPPTS 890 series to identify substances that have the potential to interact with the estrogen, androgen, or thyroid hormone pathways. The H295R Steroidogenesis Assay is intended to identify xenobiotics that affect the steroidogenic pathway beginning with the sequence of reactions occurring after the gonadotropin hormone receptors through the production of testosterone and estradiol. In this assay, the test substances are exposed to the H295R cells at seven concentrations for approximately 48 hours. At the end of the exposure period, the medium is removed from the cells, cell viability is monitored using an MTT assay, and effects on testosterone and/or estradiol are monitored using HPLC/MS/MS analysis of the H295R media samples. Forskolin, an inducer of testosterone (T) and estradiol (E2), and prochloraz, an inhibitor of T and E2, are used as the positive controls for the assay. The negative control is the appropriate vehicle control.

8. Test & Control Substance(s)

Note: A certificate of analysis for the test substances will be provided by the sponsor and will be stored with the study data and appended to the study report. Confirmation of the identity of the test substances, characterization and stability will be verified by the sponsor or sponsor's designee. Test substance will be either returned to the sponsor or destroyed following finalization of the study report.

Certificates of analysis for the negative and positive control substances, prochloraz and forskolin, respectively, will be provided by the vendor, stored in the study data and appended to the study report.

9. Preparation of Test Substance

Test substance(s) will be formulated in appropriate vehicle or dimethyl sulfoxide (DMSO). The total volume of test substance formulation used in each assay will result in no more than 0.1% DMSO in the final dosing solution in order to minimize the potential of the solvent to inhibit the cell based assay. Fresh dilutions of the stock solution will be prepared in the same solvent as the stock solution on the day of use. Dose concentrations of the test substances and control substances will not be verified.

Test Substances:

Test Substance: 2-Ethylhexyl-p-dimethyl-aminobenzoate (Padimate O)

CAS No. 21245-02-3

Source:	Sigma-Aldrich
Lot/Batch No.:	MKBF0590V
Formula:	$(CH_3)_2NC_6H_4CO_2CH_2CH(C_2H_5)(CH_2)_3CH_3$
Description:	Colorless liquid
Purity:	98.1%
Test Substance:	2-Phenyl-5-benzimidazolesulfonic acid (Ensulizole)
CAS No.	27503-81-7
Source:	Sigma-Aldrich
Lot/Batch No.:	05117JE
Formula:	$C_{13}H_{10}N_2O_3S$
Description:	White powder
Purity:	99.6%
Test Substance:	3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)
CAS No.	118-56-9
Source:	Spectrum Chemical Mfg. Corp
Lot/Batch No.:	YT0976
Formula:	$C_{16}H_{22}O_3$
Description:	Colorless to light yellow liquid
Purity:	99.3%
Test Substance:	Butyl-methoxydibenzoylmethane (Avobenzone)

CAS No.	70356-09-1
Source:	Universal Preserv-A-Chem Inc.
Lot/Batch No.:	L802809
Formula:	$C_{20}H_{22}O_3$
Description:	Off White to Yellowish Crystalline Powder
Purity:	98.5%

Test Substance Preparation:

- A 200 mM stock solution of the test substances will be prepared in DMSO, and mixed thoroughly. For the test substance, this will be the Stock 1 Test Solution.
- The test substance stock solutions will be diluted as follows:
- The 200 mM Stock 1 Test Solution will be diluted 1:10 to make the Stock 2 solution (20 mM). The 1:10 serial dilutions will be continued until a total of seven dilutions have been prepared (Stock 1 – Stock 7).

10. Control Substances

All information regarding supplier, lot numbers and purity will be included in the study reports.

Forskolin

CAS No: 66575-29-9

Molecular Formula/Weight: MW=410.50

Supplier/source: Sigma Chemical

Prochloraz

CAS No: 67747-09-5

Molecular Formula/Weight: MW=376.67

Supplier/source: Sigma Chemical

Note: Copies of the Certificates of analysis for Forskolin and Prochloraz will be maintained in the study binder.

Control Substance Preparation:

- A 20 mM stock solution of forskolin and prochloraz will be prepared in DMSO.
- The control substance stock solutions will be diluted as follows:
- Forskolin: The 20 mM solution will be diluted 1:10 to make a 2 mM solution.
- Prochloraz: The 20 mM solution will be diluted 1:10 to make a 2 mM solution. The 2 mM solution 1:10 to make a 0.2 mM solution.

11. Identification of Test System

The cells used for the steroidogenesis assay will be the H295R human adrenocortical carcinoma cells (ATCC CRL-2128).

The cells used in the study will be appropriately labeled and will be identified by cell type and passage number. Bias is not a factor in this test system.

12. Justification of Test System

The H295R human adrenocortical carcinoma cell line will be used in the study as required in the test guideline (OPPTS 890.1550). This cell line expresses genes that encode for the key enzymes in steroidogenesis.

13. Cell Culture

After initiation from an ATCC batch, cells will be grown for five passages. Passage five cells will then be frozen in liquid nitrogen. Cells started from frozen batches will be cultured for at least four additional passages before they are used to conduct the assay. The maximum passage number used in the assay will be passage 10.

The H295R cells will be maintained according to the Endocrine Disruptor Screening Program Test Guidelines OPPTS 890.1550: Steroidogenesis and CeeTox SOP 3039. The cells will be grown in supplemented media containing a DMEM:F12 media base with ITS + Premix (insulin, transferrin, selenium, BSA, and linoleic acid) and Nu-Serum.

Note: The passage number used in the assay will be provided in the report.

14. Quality Control Plate Requirements

Quality Control Plate

A quality control (QC) plate will be assayed with each experiment. The QC plate contains the controls for the assay. The quality control (QC) plate will be incubated, exposed to control substances and assessed in the same manner as test plates. The cells will be exposed with a known inducer (forskolin) and inhibitor (prochloraz) of estradiol (E2) and testosterone (T) synthesis. Exposure concentrations will be 1 and 10 µM for forskolin and 1 and 0.1 µM for prochloraz (see Table 1):

Table 1: Example Quality Control Plate Layout – 48-well Plate

	1	2	3	4	5	6	7	8
A	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH
B	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH
C	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH
D	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH
E	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH
F	Blank	Vehicle	Forskolin 1 µM	Forskolin 10 µM	Prochloraz 1 µM	Prochloraz 0.1 µM	Background	Vehicle + MeOH

Blank wells receive medium containing 22R-hydroxycholesterol. Background wells receive medium only.
Methanol (MeOH) is used as a positive control for toxicity

The QC plate criteria are presented in Table 2.

Table 2: Quality Control Plate Criteria

	Testosterone	**Estradiol**
Basal Production	≥ 5 times method detection limit	≥ 2.5 times method detection limit
Induction (10 µM forskolin)	≥ 2 times solvent control	≥ 7.5 times solvent control
Inhibition (1 µM prochloraz)	≤ 0.5 times solvent control	≤ 0.5 times solvent control

If basal E2 production does not meet the minimum basal production level specified in Table 2, 22-R hydroxycholesterol may be added to the supplemented medium to increase basal production. A 22R-hydroxycholesterol stock will be prepared in ethanol and added to the supplemented medium. The final ethanol concentration in the supplemented medium with 22R-hydroxycholesterol will be 0.025%.

Hormone Measurement System Evaluation

Analysis of the production of testosterone and estradiol by H295R cells will be conducted by HPLC/MS/MS by OpAns, LLC.

15. Test Conditions and Test Methods

Plating and Pre-Incubation of Cells

The H295R cells will be plated in supplemented media containing 10 µM 22R-hydroxycholesterol at a density of approximately 126,000 cells/well in a 48-well plate (approximately 420 µL of cell suspension per well will be added to the plate) to achieve approximately 50-60% confluency in the wells at approximately 24 hours. The plated cells will be pre-incubated for approximately 24 hours in an incubator at ~37°C with ~5% CO_2.

Exposure of Cells

Cells will be cultured and plated in 48-well plates according to the cell culture procedures described above.

Prior to exposure, a mastermix will be prepared of each test substance stock solution (section 9.0) by adding 2 µl of the test substance stock solution to 3.998 ml of supplemented medium containing 10 µM 22R-hydroxycholesterol. Also, a mastermix will be prepared containing 2 µl of DMSO and 3.998 ml of supplemented medium containing 10 µM 22R-hydroxycholesterol. This solution will be used to expose the vehicle control wells. The final DMSO concentration in all solutions will be 0.05%. The final ethanol concentration (resulting from the preparation of a 22R-hydroxycholesterol stock in ethanol) in the solutions will be 0.025%. At the time of exposure, the test substance dilutions will be visually observed for precipitation. Additionally, test substance solubility will be evaluated by nephelometry before and after the exposure period. The final dilutions into supplemented medium yield final exposure concentrations of 100, 10, 1, 0.1, 0.01, 0.001, and 0.0001 µM for each test substance.

After the initial 24 hour pre-incubation of the cells, the plates will be removed from the incubator and checked for attachment and morphology under the microscope prior to test substance exposure. Observations will be recorded.

The media will be removed from each well and the test substance or vehicle mastermix prepared above will be added by pipetting (420 µL/well of the appropriate mastermix to the appropriate wells – example dosing scheme is presented in Table 3). Plates will then be returned to an approximately 37°C, ~5% CO_2 incubator.

Table 3: Example Dosing Scheme for Exposure of H295R Cells to Test Substances in a 48-Well Plate

	1	2	3	4	5	6	7	8
A	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM
B	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM
C	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM
D	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM
E	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM
F	DMSO	Stock 1 100 µM	Stock 2 10 µM	Stock 3 1 µM	Stock 4 0.1 µM	Stock 5 0.01 µM	Stock 6 0.001 µM	Stock 7 0.0001 µM

Plates will be incubated at approximately 37°C with 5% CO_2 in an incubator for approximately 48 hours.

After the approximately 48 hour exposure, plates will be removed from the incubator and every well checked under the microscope for cell condition. Observations will be recorded. Media will be collected from each well into two equal aliquots and transferred into two separate sets of tubes or plates. Media will be frozen at approximately -80°C until further processing (HPLC/MS/MS analytical detection methods for T and E2). Test substance solubility will also be evaluated by nephelometry after the 48 hour exposure period.

Immediately after removing media, an MTT cell viability test will be conducted on each exposure plate.

MTT Viability Assay

Following the full ~48 hour exposure period and media collection, an MTT assay will be performed by adding MTT medium (0.5 mg/ml) to each well. The plates will be returned to a ~37°C, ~5% CO_2, humidified incubator for approximately a 3 hour incubation. After this incubation, the plates will be removed from the incubator and blue formazan salt will be extracted with isopropanol per well for ~20 minutes at room temperature with shaking. After the extraction, the optical density of the extracted formazan will be

determined using a spectrophotometer (570 nm). Viable cells will have the greatest amount of MTT reduction and hence the highest absorbance values. Relative cell viability will be calculated for each tissue as a percentage of the mean of the vehicle control wells.

Hormone Measurement

HPLC/MS/MS

Samples will be split into two portions. At least one aliquot will be shipped to OpAns, LLC for determination of estradiol and testosterone levels. Hormone concentrations will be measured using bioanalytical methods validated by OpAns, LLC. References to the validated methods will be included in the final report.

16. Test Results and Data Analysis

Data Processing

Results will be expressed as change in hormone production relative to the mean solvent control for the assay. Data will be expressed as mean ± standard deviation. All doses that exhibit cytotoxicity greater than 20% or for which precipitation is observed will be omitted from further evaluation.

Relative changes will be calculated using the equation below:

Relative (Fold) Change = [Hormone] in each well ÷ [Hormone] of mean vehicle control

Table 4. Data Categorization Parameters for the Analysis of Results obtained with the H295R Steroidogenesis Assay

Parameter	Criterion
Statistical Significance	Difference from the Solvent Control will have $p \leq 0.05$.
Dose Response	Data will be expected to follow a dose response type profile at non-cytotoxic doses, or doses that do not interfere with the hormone measurement assay (note: response can be bi-phasic such as an increase at lower and a decrease at higher doses, but changes randomly observed only at a few concentrations within the dose range are to be excluded)
Solubility	The results at concentrations for which cloudiness or a precipitate will be observed will not be included.
Cell Viability	Only non-cytotoxic concentrations (>80% viability) will be included.

Description of Proposed Statistical Methods

Prior to conducting statistical analyses, Dixon Q test for outlier identification and rejection will be performed followed by the evaluation of normality and variance homogeneity. Normality will be evaluated using standard probability plots or another appropriate statistical method (e.g., Shapiro-Wilk's test). If the data are not normally distributed, the data will be transformed to approximate a normal distribution. If the data are normally distributed or approximate normal distribution, differences between substance treatments and solvent controls (SCs) will be analyzed using a parametric test (e.g., Dunnett's Test). If data are not normally distributed, an appropriate non-parametric test will be used (e.g., Kruskal Wallis, Steel's Many-one rank test). Differences will be considered significant at $p \leq 0.05$. Statistical analyses will be performed using Unistat 6.0 Light for Excel.

A summary of criteria for the evaluation of data is provided in Table 4.

Data Interpretation

A test substance will be judged to be positive if the fold induction is statistically different from the vehicle control. Statistical differences will be considered significant at $p \leq 0.05$ using the analyses described above. Results exceeding the limits of solubility or at cytotoxic concentrations will be excluded from the statistical analysis.

17. Final Study Report

The data to be reported will be determined per SOP and will include (but will not be limited to) the following information: assay date and run number, laboratory personnel involved in the study, reference/test substance information (including but not limited to substance name, code, molecular weight, concentrations tested, notes regarding solubility), and data summary and interpretation.

The Principal Investigator from OpAns, LLC will provide a signed report describing the sample analysis as well as all concentration results. The entire Principal Investigator report will be included as an appendix to the study report.

18. Alterations of the Study Design

Alterations of this protocol may be made as the study progresses. No changes in the protocol will be made without the specific written request or consent of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, CeeTox will honor such a change. However, written authorization will be obtained to document this verbal request. All protocol amendments with justifications will be documented, signed and dated by the Study Director and the Sponsor's Representative. A copy of the protocol

and all amendments will be issued to the Sponsor and originals will be placed in the study binder.

19. Record Retention and Archiving

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

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

20. Test Substance Disposition

Test substance will be either returned to the sponsor or destroyed following finalization of the study report.

CeeTox

Protocol Amendment 1

Study Number: 9070-100794STER

Title of Study to be Amended: H295R Steroidogenesis Assay

Reason for Amendment to Protocol: Ensulizole was insoluble in DMSO at the 200 mM stock concentration specified in the protocol. This amendment modifies the test substance preparation and final exposure concentrations **for ensulizole only**.

Changes:

Section 9
Section 9 specifies the following test substance preparation procedure:

Test Substance Preparation:

- A 200 mM stock solution of the test substances will be prepared in DMSO, and mixed thoroughly. For the test substance, this will be the Stock 1 Test Solution.

- The test substance stock solutions will be diluted as follows:

- The 200 mM Stock 1 Test Solution will be diluted 1:10 to make the Stock 2 solution (20 mM). The 1:10 serial dilutions will be continued until a total of seven dilutions have been prepared (Stock 1 – Stock 7).

For **ensulizole only**, the procedure will be modified as follows:

- A 20 mM stock solution of the test substance will be prepared in DMSO, and mixed thoroughly. For the test substance, this will be the Stock 1 Test Solution.

- The test substance stock solution will be diluted as follows:

- The 20 mM Stock 1 Test Solution will be diluted 1:10 to make the Stock 2 solution (2 mM). The 1:10 serial dilutions will be continued until a total of seven dilutions have been prepared (Stock 1 – Stock 7).

Section 15 and Table 3

Section 15 and Table 3 specify that the final dilutions into supplemented medium yield final exposure concentrations of 100, 10, 1, 0.1, 0.01, 0.001, and 0.0001 µM for each test substance.

For **ensulizole only**, the final exposure concentrations will be 10, 1, 0.1, 0.01, 0.001, 0.0001, and 0.00001 µM.

Signatures

[redacted]

Study Sponsor

4/11/13
Date

[redacted]

Study Sponsor

4/16/13
Date

[redacted]

Study Monitor

10 APRIL 2013
Date

CeeTox, Inc.

[redacted]

Study Director

17 April 2013
Date