CeeTox
In vitro models to predict toxicity

Androgen Receptor Binding (Rat Prostate Cytosol)

Final Report

DATA REQUIREMENT(S): OPPTS 890.1150 (2009)

AUTHOR(S): [redacted]

STUDY COMPLETION DATE: April 18, 2013

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STATEMENT OF DATA CONFIDENTIALITY CLAIMS

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GOOD LABORATORY PRACTICE COMPLIANCE STATEMENT

Study Number: 9070-100794ARB

Study Title: Androgen Receptor Binding (Rat Prostate Cytosol)

I, the undersigned, hereby declare that this study was performed in accordance with the Environmental Protection Agency (EPA) Good Laboratory Practice (GLP) regulations Title 40 Part 160 with the exception of section 160.113. Dose concentrations of test and control substances will not be 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

18 April 2013

Date

FLAGGING STATEMENT

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QUALITY ASSURANCE STATEMENT

Study Title: Androgen Receptor Binding (Rat Prostate Cytosol)

Study Number: 9070-100794ARB

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
20Dec12	Draft Protocol Review	20Dec12	20Dec12
11Feb13	Test Substance Prep and Day 1 Assay	18Feb13	18Feb13
15Feb13	Day 2 Assay	18Feb13	18Feb13
11Mar13	Data Binder Review	11Mar13	11Mar13
12Mar13	Draft Report Review	12Mar13	12Mar13

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

[redacted] 18Apr2013

Date

Quality Assurance Auditor
4717 Campus Drive
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GENERAL INFORMATION

Contributors

The following contributed to this report in the capacities indicated:

Name	Title
[redacted]	Director of Project Management
	Senior Scientist
	Scientist
	Laboratory Manager
	Study Director

Study Dates

Study initiation date: January 16, 2013
Experimental start date: February 11, 2013
Experimental termination date: February 17, 2013
Study termination date: April 18, 2013

Deviations from the Protocol

See Appendix 3. There were three deviations however they 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 ensulizole, avobenzone, homosalate and padimate-O to interact with the androgen receptors (ARs) isolated from rat prostates.

Assessments of precipitation were conducted during each run in order to identify a suitable top concentration of ensulizole, avobenzone, homosalate and padimate-O for use in the binding assays. The concentrations assessed were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M.

The final concentrations of ensulizole assessed in the binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M for all three valid independent runs (11-February-2013, 14-February-2013 and 16-February-2013), while the final concentrations of avobenzone, homosalate and padimate-O assessed in the binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} because of precipitation of the test substances at 10^{-4} and 10^{-3} M.

Three independent runs of the AR binding assay were conducted. All concentrations were tested in replicates of 3. In addition, solvent control tubes (6 replicates) were prepared to assess total binding. These replicates included the radioligand, cytosol (containing the ARs) and solvent but without the competitor R1881. The total binding tubes allowed for the identification of maximal binding of [^{3}H]-R1881. Non-specific binding (NSB) was also assessed in replicates of 6 by determining the [^{3}H]-R1881 bound in the presence of 100-fold excess unlabeled R1881. NSB was subtracted from the data, normalized to total binding and presented as % specific binding. Finally, 30 µL of [^{3}H]-R1881 was added to scintillation vials (n=6) in order to determine both total radioligand added and to calculate the percentage of total radioligand added to the tube that is bound to ARs. The duration of incubation at approximately 4°C was 16-20 hours. A complete concentration response curve for the positive control R1881 and the weak positive control (wPC) dexamethasone was run each time the binding assay was performed.

1.2 Results

The suitable top concentration of ensulizole was 10^{-3} M for use in all three valid independent runs (11-February-2013, 14-February-2013 and 16-February-2013) while the suitable top concentration of avobenzone, homosalate and padimate-O for use in all three valid independent runs was 10^{-5} M as precipitation was seen at 10^{-4} and 10^{-3} M.

In the first valid independent run (11-February-2013), the mean specific binding was > 75% at every soluble concentration tested for ensulizole, avobenzone, homosalate and padimate-O resulting in a classification of "non-binder" for all four test substances. The mean specific binding for avobenzone, homosalate and padimate-O at 10^{-4} M and 10^{-3} M was not assessed because precipitation was observed at these concentrations. The weak positive control dexamethasone had a $LogIC_{50}$ of -4.5 M while the $LogIC_{50}$ of R1881 was -9.9 M.

In the second valid independent run (14-February-2013), the mean specific binding was > 75% at every soluble concentration tested for avobenzone, ensulizole and homosalate resulting in a classification of "non-binder" for these three test substances. The mean specific binding for avobenzone, homosalate and padimate-O at 10^{-4} M and 10^{-3} M was not assessed because precipitation was observed at these concentrations. The mean specific binding for padimate-O was > 75% at every soluble concentration tested except for 10^{-8} M (44.8%) and 10^{-6} M (75.0%). A Dixon-Q outlier test can eliminate one of the three replicates in the 10^{-6} M data set, resulting in a mean specific binding value of 102.4%. Additionally, the replicates at 10^{-8} M are substantially varied, exhibiting specific binding of -0.3% (replicate 1), 54.6% (replicate 2) and 80.0% (replicate 3). Because all three replicates are so varied, the Dixon-Q outlier test will not allow removal of one or more of the replicates in the analysis. However, these inconsistencies between replicates suggest a loss of all or a part of the HAP pellet during the final extraction and washing steps. Since all soluble concentrations, both higher and lower than 10^{-8} M, exhibit mean specific binding of > 75%, padimate-O is also classified as a "non-binder" for this run. The weak positive control dexamethasone had a $LogIC_{50}$ of -4.5 M while the $LogIC_{50}$ of R1881 was -9.9 M.

Finally, in the third valid independent run (16-February-2013), the mean specific binding was > 75% at every soluble concentration tested for ensulizole, avobenzone, homosalate, and padimate-O, resulting in the classification as a "non-binder" for all four test substances. The mean specific binding for avobenzone, homosalate and padimate-O at 10^{-4} M and 10^{-3} M was not assessed because precipitation was observed at these concentrations. The weak positive control dexamethasone had a $LogIC_{50}$ of -4.6 M while the $LogIC_{50}$ of R1881 was -10.0 M.

The mean relative binding affinity, or RBA (calculated by dividing the $LogIC_{50}$ of the control/test material by the $LogIC_{50}$ of the positive control R1881) was 0.5 for dexamethasone. As ensulizole, avobenzone, homosalate and padimate-O were not classified as an overall "binder" (mean specific binding $\geq$ 50%), the RBA could not be calculated.

1.3 Conclusion

All four test materials, ensulizole, avobenzone, homosalate and padimate-O are classified as "non-binders" of the AR in all three independent runs and thus have a final classification of "non-binder."

2.0 INTRODUCTION

2.1 Purpose

The objective of this study was to evaluate the ability of ensulizole, avobenzone, homosalate and padimate-O to interact with the androgen receptors (ARs) isolated from rat prostates. The AR contains a highly specific hormone-binding domain (HBD) that is relatively well conserved across species. Upon binding endogenous androgens to the HBD, the AR enters the nucleus and binds to specific sites in the genome called androgen response elements (AREs). Once bound to the ARE, the AR forms a homodimer with another AR, thereby controlling gene expression.

This assay was used to provide information on the ability of a compound to interact with the androgen receptors (ARs) isolated from rat prostates. This assay is not intended to be used to show that the interaction is, specifically, one-site competitive binding, or to precisely characterize the strength of the binding interaction. It therefore may not be appropriate to use in quantitative structure-activity relationship (SAR) model development for androgen receptor binding without further refinement. This assay is intended to be used as one part of a screening program that includes other assays, to detect substances that can potentially interact with the androgen hormonal system.

The results of this study are intended to be used in conjunction with results from other Tier 1 screening studies (OPPTS 890 test guideline series) that constitute the full screening battery under the Endocrine Disruptor Screening Program (EDSP). Together, the results from the screening battery will be used by the US EPA to identify substances that have the potential to interact with the androgen, estrogen or thyroid system. Results of the Tier 1 screening battery, along with other scientifically relevant information, are to be used in a weight-of-evidence determination of a substance's potential to interact with these systems. The fact that a substance may interact with a hormone system does not mean that when the substance is used, it will cause adverse effects in humans or ecological systems. The Tier 1 battery is intended for screening purposes only and should not be used for endocrine classification or risk assessment.

2.2 Regulatory Citations

OPPTS 890.1150: Androgen Receptor Binding (Rat Prostate Cytosol). 2009 (now referred to as OCSPP though the guideline is still titled as OPPTS).

3.0 MATERIALS AND METHODS

All materials and methods described in this report are in reference to the three valid independent runs (11-February-2013, 14-February-2013 and 16-February-2013) only.

3.1 Test Substance

3.1.1 Test Substance Details

Test Substance Name:	2-Phenyl-5-benzimidazolesulfonic Acid (Ensulizole)
Test Substance Supplier:	Aldrich
CAS Number:	27503-81-7
Description:	White to off white powder
Solvent Used:	Dimethyl sulfoxide
Batch Number:	05117JE
Expiry Date:	Not provided
Purity:	99.6%
Molecular Formula:	$C_{13}H_{10}N_2O_3S$
Molecular Weight:	274.30 g/mol
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 4.

Test Substance Name:	Butyl-methoxydibenzoylmethane (Avobenzone)
Test Substance Supplier:	Universal-Preserv-A-Chem, Inc.
CAS Number:	70356-09-1
Description:	Off white to yellowish crystalline powder
Solvent Used:	Dimethyl sulfoxide
Batch Number:	L802809
Expiry Date:	Not provided
Purity:	98.5%
Molecular Formula:	$C_{20}H_{22}O_3$
Molecular Weight:	310.39 g/mol
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 4.

Test Substance Name:	3, 3, 5-Trimethlycyclohexyl Salicylate (Homosalate)
Test Substance Supplier:	Spectrum
CAS Number:	118-56-9
Description:	Colorless to light yellow liquid
Solvent Used:	Dimethyl sulfoxide
Batch Number:	YT0976
Expiry Date:	Not provided
Purity:	99.3%
Molecular Formula:	262.34 g/mol
Molecular Weight:	$C_{16}H_{22}O_3$
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 4.

Test Substance Name:	2-Ethylhexyl-P-Dimethyl-Aminobenzoate (Padimate-O)
Test Substance Supplier:	Aldrich
CAS Number:	21245-02-3
Description:	Yellowish liquid
Solvent Used:	Dimethyl sulfoxide
Batch Number:	MKBF0590V
Expiry Date:	Not provided
Purity:	98.1%
Molecular Formula:	277.40 g/mol
Molecular Weight:	$C_{17}H_{27}NO_2$
Storage Conditions:	Room Temp. (eg. ambient)

A certificate of analysis for the test substance is presented in Appendix 4.

The reference compound R1881 (CAS# 965-93-5) was purchased from Sigma Aldrich (St. Louis, MO) and was 99% pure. The catalog number was R0908 and the lot number was 112M4617V.

The weak positive control dexamethasone (CAS# 50-02-2) was purchased from Sigma Aldrich (Buchs, Switzerland) and was 98.9% pure. The catalog number was D1756 and the lot numbers were 1419230.

The radioligand [^{3}H]-R1881 was purchased from Perkin-Elmer (Boston, MA) and had a specific activity (SA) of 85.1 Ci/mmol on the certification date (10-October-2012). The catalog number was NET590 and the lot number was 1001606. The $SA_{adjusted}$ was 83.5 Ci/mol for the first two valid independent runs (11-February-2013 and 14-February-2013) and 83.4 Ci/mol for the third valid independent run (16-February-2013).

3.1.2 Vehicle Selection

Dimethyl sulfoxide (DMSO) is one of the recommended solvents according to the EPA guideline (OPPTS 890.1150) and was selected as a suitable vehicle for ensulizole, avobenzone, homosalate and padimate-O. Ensulizole solutions with a concentration of up to 10^{-3} M, and avobenzone, homosalate and padimate-O solutions with a concentration of up to 10^{-5} M (the limit concentration for the assay) were prepared while limiting the final concentration of DMSO in the assay medium to ~3.2% (v/v). R1881 and dexamethasone were prepared on 11-February-2013, used for the first run, then frozen as aliquots and thawed on the day of the assay for use in the second and third valid independent runs. The test substances were prepared fresh on the day of the assay for all three valid independent run.

3.1.3 Test Substance Preparation

Vehicle (DMSO) was kept at the same concentration for the controls and for the test substances. DMSO was tested as a vehicle control with the reference chemical and reference controls for the run as well. All concentrations of ensulizole, avobenzone, homosalate and padimate-O were kept at approximately 3.2% final DMSO concentration. The dose

concentrations of ensulizole, avobenzone, homosalate and padimate-O were not verified using analytical methods.

Serial dilutions of test chemicals were prepared in DMSO to yield the final concentrations indicated below:

Example Dilution Procedure for ensulizole, avobenzone, homosalate and padimate-O.

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted test chemical	Diluted test chemical concentration	*Final test chemical concentration in AR assay tube
TC1	Use 300 µl of stock test chemical (100 mM)	700 µl	1 ml	3×10^{-2} M	1×10^{-3} M
TC2	Use 100 µl of dilution TC1 (50 mM)	900 µl	1 ml	3×10^{-3} M	1×10^{-4} M
TC3	Use 100 µl of dilution TC2 (5 mM)	900 µl	1 ml	3×10^{-4} M	1×10^{-5} M
TC4	Use 100 µl of dilution TC3 (500 µM)	900 µl	1 ml	3×10^{-5} M	1×10^{-6} M
TC5	Use 100 µl of dilution TC4 (50 µM)	900 µl	1 ml	3×10^{-6} M	1×10^{-7} M
TC6	Use 100 µl of dilution TC5 (5 µM)	900 µl	1 ml	3×10^{-7} M	1×10^{-8} M
TC7	Use 100 µl of dilution TC6 (500 nM)	900 µl	1 ml	3×10^{-8} M	1×10^{-9} M
TC8	Use 100 µl of dilution TC7 (50 nM)	900 µl	1 ml	3×10^{-9} M	1×10^{-10} M

*Final concentration of ensulizole, avobenzone, homosalate and padimate-O in assay tube when 10 µl of diluted concentration is used in a total volume of 300 µl.

3.1.4 Positive and Weak Positive Reference Control Preparation

The positive control, R1881, strongly binds ARs and was included to ensure that the run was properly performed and to allow an assessment of variability in the conduct of the assay across time. Final concentrations of unlabeled R1881 ranged from 1×10^{-6} to 1×10^{-11} M as described below. Fresh 10 mM R1881 stock was diluted 1:10 (1 mM R1881 solution) and then serial dilutions of the reference standard were performed in DMSO (final concentration of approximately 3.2%).

Example Dilution Procedure for R1881

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of R1881	Diluted R1881 concentration	*Final R1881 concentration in AR assay tube
N/A	Use 100 µl of stock R1881 (10 mM)	900 µl	1 ml	1×10^{-3} M	N/A
NSB1	Use 30 µl of stock R1881 (1 mM)	970 µl	1 ml	3×10^{-5} M	1×10^{-6}
S2	Use 100 µl of dilution NSB1 (30 µM)	900 µl	1 ml	3×10^{-6} M	1×10^{-7}
S3	Use 100 µl of dilution S2 (3 µM)	900 µl	1 ml	3×10^{-7} M	1×10^{-8}
S4	Use 100 µl of dilution S3 (300 nM)	900 µl	1 ml	3×10^{-8} M	1×10^{-9}
S5	Use 100 µl of dilution S4 (30 nM)	900 µl	1 ml	3×10^{-9} M	1×10^{-10}
S6	Use 100 µl of dilution S5 (3 nM)	900 µl	1 ml	3×10^{-10} M	1×10^{-11}

*Final concentration of control in assay tube when 10 µl of diluted concentration is used in a total volume of 300 µl.

The weak positive control was dexamethasone. A 30 mM stock was prepared in DMSO and serially diluted as described below. The concentration range tested for the weak positive control was from 1 x 10^{-3} to 1 x 10^{-10} M with DMSO kept at approximately 3.2%**.**

Example Dilution Procedure for Dexamethasone

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted positive control	Weak Positive Control Concentration	
				Diluted	*Final in AR assay tube
P1	Use stock positive control (30 mM)	N/A	1 ml	3 x 10^{-2} M	1 x 10^{-3} M
P2	Use 100 µl of stock positive control (30 mM)	900 µl	1 ml	3 x 10^{-3} M	1 x 10^{-4} M
P3	Use 100 µl of P2 (3 mM)	900 µl	1 ml	3 x 10^{-4} M	1 x 10^{-5} M
P4	Use 100 µl of P3 (300 µM)	900 µl	1 ml	3 x 10^{-5} M	1 x 10^{-6} M
P5	Use 100 µl of P4 (30 µM)	900 µl	1 ml	3 x 10^{-6} M	1 x 10^{-7} M
P6	Use 100 µl of P5 (3 µM)	900 µl	1 ml	3 x 10^{-7} M	1 x 10^{-8} M
P7	Use 100 µl of P6 (300 nM)	900 µl	1 ml	3 x 10^{-8} M	1 x 10^{-9} M
P8	Use 100 µl of P7 (30 nM)	900 µl	1 ml	3 x 10^{-9} M	1 x 10^{-10} M

*Final concentration of control in assay tube when 10 µl of diluted concentration is used in a total volume of 300 µl.

3.2 Solubility/Precipitation Assay

The limit of test substance solubility was determined by laser based light scattering. The test substance was prepared in the TEDG buffer alone (no cytosol) at the final exposure concentrations and added to wells of a 96-well plate. The samples were assessed using a NEPHELOstar nephelometer (BMG LabTech, Ortenberg, Germany).

3.3 Rat Prostate Cytosol

102 prostate glands from 90-day old (< 1 day since castration) male Sprague-Dawley rats were purchased from Charles River Laboratories. Cytosol was prepared and verified at CeeTox per EPA guideline OPPTS 890.1150 and CeeTox SOP 2055 for use on this study. As the cytosol was prepared in large batches for use in multiple assays of different test substances, data related to preparation and saturation binding of the cytosol are maintained separate from this study; the pertinent information is available in Appendix 2.

3.4 Stock Solution Preparation

A 200 mM EDTA stock solution was prepared and stored at approximately 4°C. A 1 M sodium molybdate solution was also prepared along with a 1 M Tris buffer (pH adjusted to

7.4). These solutions were then used to prepare Low-salt TEDG Buffer (10 mM Tris, 1 mM sodium molybdate, 1.5 mM EDTA, 10% glycerol and 1 mM DTT [added immediately before use], pH 7.4 [cooled to approximately 4°C before adjusting to pH 7.4 and stored at approximately 4°C up to 3 months]).

A 600 µM stock solution of triamcinolone acetonide was prepared in 100% ethanol and diluted/aliquoted into 60 µM solutions and stored at approximately -20°C.

The 60% hydroxyapatite (HAP) slurry was prepared one day before use. The HAP was gently mixed with 50 mM buffer in a graduated cylinder, and refrigerated for approximately 2 hours at approximately 4°C. The HAP was then washed three times as follows. The supernatant was removed and the HAP was resuspended again in 50 mM Tris buffer (approximately 4°C). The slurry was mixed gently and allowed to settle for approximately 2 hours at approximately 4°C. After the third wash, the HAP slurry settled overnight (at least 8 to 10 hours at approximately 4°C).

The next day (day of use), the volume of HAP on the graduated cylinder was noted. The supernatant was removed and the HAP was resuspended to a final volume of 60% HAP and 40% cold 50 mM Tris buffer. The HAP slurry was well-suspended and ice-cold when used in the separation procedure.

3.5 Assays

3.5.1 Working Assay Buffer Preparation

Summary Table of Assay Conditions

		Competitive Binding Assay Protocol
Source of receptor		Rat prostate cytosol
Concentration of radioligand		1 nM
Concentration of receptor		Sufficient to bind 10-15% of radioligand
Concentration of test substance (as serial dilutions)		100 pM to 1 mM
Temperature		~4°C
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10% (v/v)
	Protease Inhibitor	0.5% (v/v)
	DTT	1 mM
	Sodium Molybdate	1 mM

On the day of assay, the Working Assay Buffer, or TEDG+PI buffer (10 mM Tris, 1 mM sodium molybdate, 1.5 mM EDTA, 10% glycerol and 1 mM DTT, 0.5% Protease Inhibitor (v/v), pH 7.4) was prepared using the TEDG buffer.

3.5.2 [^{3}H]-R1881 Preparation

[^{3}H]-R1881 was prepared on the day of assay. The specific activity was adjusted for decay over time prior to performing dilutions. The specific activity was calculated on the day of the assay using the following equation:

$SA_{adjusted}$ (Fraction Isotope Remaining) = SA * $e^{-Kdecay*Time}$

SA is the specific activity on the packaging date.
Kdecay is the decay constant for tritium (equal to 1.54 x 10^{-4}/day).
Time = days since the date on the stock bottle from the manufacturer.

The [^{3}H]-R1881 was diluted with TEDG + PI buffer so that each assay tube contained 1 nM final concentration of [^{3}H]-R1881 using the following procedure:

The specific activity was converted from Ci/mmole to nM. If SA = X Ci/mmole, and Y = concentration of radiolabel, then X Ci/mmole was converted to nM and the SA activity adjusted for decay over time by the following conversion:

(Y mCi/ml / X Ci/mmole) * 1 Ci/1000 mCi * 10^6 nmole/mmole * 1000 ml/L = (Y/X) * 10^6 nM

A 10 nM diluted stock of the [^{3}H]-R1881 was prepared so that 30 µl in a total volume of 300 µl per assay tube will give a final concentration of 1 nM. The 10 nM [^{3}H]-R1881 was kept on ice until standards, test chemicals, and assay tubes were prepared.

3.5.3 Assay Preparations

Glass 12 x 75 mm tubes were used for the assay. 30 µl of 10 nM [^{3}H]-R1881 (1 x 10^{-8} M) and 50 µl triamcinolone acetonide (60 µM working solution) were added to all tubes. For the 3 tubes at the beginning of assay and at the end of assay, 100X inert R1881 (30 µl of 1 µM) was also added. These were the nonspecific binding tubes. The tubes were placed in a speed-vac and dried. An aliquot of cytosol was thawed on ice and diluted to the pre-determined optimal protein concentration.

3.5.4 Individual Tubes

For the assay tubes, 10 µl of each concentration of test substance and control was added, followed by 300 µl of the diluted cytosol. The temperature of the tubes and contents were kept at approximately 4°C prior to the addition of the cytosol. The assay tubes were vortexed after additions and incubated at approximately 4°C for 16 to 20 hours on a rotator.

3.5.5 Separation of Bound [^{3}H]-R1881 From Free [^{3}H]-R1881

The AR assay tubes were removed from the rotator and placed in an ice-water bath. A repeating pipette was used to add approximately 500 µl of ice cold HAP slurry (60% in 50 mM Tris buffer) to fresh new 12 x 75 mm glass assay tubes. 100 µl of each incubation tube was transferred to the appropriate labelled tubes containing the HAP. The tubes were vortexed for approximately 10 seconds at approximately 5 minute intervals for a total of approximately 20 minutes with tubes remaining in the ice-water bath between vortexing. Following the vortexing step, approximately 2 ml of the cold 50 mM Tris buffer was added, quickly vortexed, and centrifuged at approximately 4°C for approximately 3 minutes at 700 x g. After centrifugation, the supernatant containing the free [^{3}H]-R1881 was

immediately decanted and discarded. The HAP pellet contained the androgen receptor bound [^{3}H]-R1881. Approximately 2 ml of ice-cold 50 mM Tris buffer was added to each tube and vortexed to resuspend the pellet. The tubes were centrifuged again at approximately 4°C for approximately 3 minutes at approximately 700 x g. The supernatant was quickly decanted and discarded. The wash and centrifugation steps were repeated three more times. After the final wash, the supernatant was decanted. The assay tubes were allowed to drain briefly for approximately 30 seconds.

3.5.6 Extraction and Quantification of [^{3}H]-R1881 Bound to AR.

Approximately 2 ml of absolute ethanol was added to each assay tube. The tubes were allowed to sit at room temperature for approximately 15 to 20 minutes, vortexing for approximately 10 seconds at approximately 5-minute intervals. The assay tubes were centrifuged for approximately 10 minutes at approximately 700 x g. The supernatant was decanted into a 20 ml scintillation vial containing approximately 14 ml scintillation cocktail (Perkin Elmer Opti-Fluor, cat# 6013199, lot# 47-12261). The vial was capped and shaken. The vials were placed in a scintillation counter (Perkin Elmer Tri-Carb 2910TR Liquid Scintillation Analyzer Model B2910) and each vial was counted for at least one minute with quench correction for determination of DPMs per vial.

Standards (^{3}H, ^{14}C and background) were used to verify accurate counting, and the liquid scintillation analyzer has an enhanced Instrument Performance Assessment (IPA) for monitoring efficiencies, backgrounds, E2/B and Chi-square values for ^{3}H and ^{14}C over the life of the instrument. The most recent IPA time and date stamped data are available on demand for reporting purposes. Each IPA printout includes instrument model, serial number, software version number and calibration standard information.

3.6 Competitive Binding Data Analysis and Interpretation

3.6.1 Analysis and Considerations

For each of the three valid independent runs of the competitive binding assays, the DPM values were added to a locked data spreadsheet (Microsoft EXCEL 2010 Version 14.0.6123.5001; Redmond, WA). The following statistics were assessed; mean specific binding (%), standard deviation (SD), standard error of the mean (SEM), percent coefficient of variation (% CV), residuals, squared residuals, and the Loge(Syx) (ie. Loge(residual standard deviation)) using XLfit (Version 5.2.0.0; Guildford, Surrey, UK). XLfit was also used for graphing the results and determining the bottom, top, and hill slope and IC_{50} (if applicable) for each curve generated.
The competitive binding assay was functioning correctly if all of the following criteria had been met, according to OPPTS 890.1150:

Increasing concentrations of unlabeled R1881 displaced [^{3}H]-R1881 from the receptor in a manner consistent with one-site competitive binding. Specifically, the curve fitted to the radioinert R1881 data points using non-linear regression descended from 90% to 10% over approximately an 81-fold increase in the concentration of the test chemicals.

Ligand depletion was minimal. Specifically, the ratio of total binding in the absence of competitor to the total amount of [^{3}H]-R1881 added per assay tube was no greater than 15%.

The parameter values (top, bottom, and slope) for R1881 and the concurrent positive control (dexamethasone) were within the tolerance bounds outlined in the OPPTS guideline and are provided below.

The solvent control substance did not alter the sensitivity or reliability of the assay. Specifically, the acceptable limit of ethanol concentration in the assay tube was 3%; the acceptable limit of DMSO concentration was ≤ 10%. All tubes must have contained equal amounts of solvent.

The test chemical was tested over a concentration range that fully defined the top of the curve (i.e. a range that showed that a top plateau was achieved), and the top was within 25 percentage points of either the solvent control or the value for the lowest concentration of the R1881 standard for that run.

Upper and Lower Limits for Parameters in Competitive Binding Assay Curves for the Standards (Radioinert R1881 and dexamethasone)

Chemical	Parameter	Lower Limit	Upper Limit
R1881 (Standard Curve)	Slope	-1.2	-0.8
	Top (%)	82	114
	Bottom (%)	-2	+2
Dexamethasone (Weak Positive)	Slope	-1.4	-0.6
	Top (%)	87	106
	Bottom (%)	-12	+12

3.6.2 Classification

The classification of a chemical as a binder or non-binder was made on the basis of the average results of three non-concurrent runs, each of which meet the performance criteria and taken together, were consistent with each other, as per OPPTS guideline 890.1150. Each run was classified as "binder," "non-binder," or "equivocal."

A run was classified as "binder" with the ARs if the lowest point on the fitted response curve within the range of the data was less than 50%.

"Percent" refers to binding of the radiolabeled R1881. Thus, "less than 50%" means that less than 50% of the radiolabeled R1881 was bound, or equivalently, that more than 50% of the radiolabeled R1881 had been displaced from the receptor. In other words, a run was classified as "binder" if a Log(IC_{50}) was obtained.

A run was classified as a "non-binder" if the lowest point on the fitted response curve within the range of the data was above 75%.

A run was classified as "equivocal" if the average lowest point on the fitted response curves within the range of the data was above 50% but below 75%.

After each run was classified, the chemical was classified by assigning the following values to each run and averaging across runs:
Binder: 2
Equivocal: 1
Non-binder: 0

Chemical classification, based on the average of all the runs performed for a chemical:
Binder: average ≥ 1.5
Equivocal: $0.5 \leq$ average < 1.5
Non-binder: average < 0.5

For example, if a chemical was tested in three runs in one lab and is determined to be interactive in 2 runs and equivocal in 1 run, to classify this chemical one would average 2, 2, and 1 = ~1.67 and the chemical would be considered a "binder" because the average was greater than 1.5.

4.0 RESULTS AND DISCUSSION

4.1 Concentration Range for the Test Substance

In order to identify a suitable top concentration for use in the binding assays, preliminary assessments of precipitation were conducted as described in Sections 3.2. The final concentrations of ensulizole, avobenzone, homosalate and padimate-O to assess precipitation were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M.

The suitable top concentration of ensulizole was 10^{-3} M for use in all three valid independent runs (11-February-2013, 14-February-2013 and 16-February-2013) while the suitable top concentration of avobenzone, homosalate and padimate-O for use in all three valid independent runs was 10^{-5} M as precipitation was seen at 10^{-4} and 10^{-3} M.

The final concentrations of ensulizole assessed in the binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6}, 10^{-5}, 10^{-4} and 10^{-3} M for all three valid independent runs (11-February-2013, 14-February-2013 and 16-February-2013), while the final concentrations of avobenzone, homosalate and padimate-O assessed in the binding assays were: 10^{-10}, 10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} because of precipitation of the test substances at 10^{-4} and 10^{-3} M.

4.2 Binding Assay Acceptance Criteria

In all three valid independent runs of the assay, increasing concentrations of unlabeled R1881 displaced [^{3}H]-R1881 from the receptor in a manner consistent with one-site competitive binding, and the ligand depletion was held below 15%. Also, the solvent did not alter the assay sensitivity or reliability. Finally, the data were within the acceptable ranges specified in Section 3.6.1.

4.3 Results

The suitable top concentration of ensulizole was 10^{-3} M for use in all three valid independent runs (11-February-2013, 14-February-2013 and 16-February-2013) while the suitable top concentration of avobenzone, homosalate and padimate-O for use in all three valid independent runs was 10^{-5} M as precipitation was seen at 10^{-4} and 10^{-3} M.

Three independent runs of the binding assay were conducted. In the first valid independent run (11-February-2013), the mean specific binding was > 75% at every soluble concentration tested for ensulizole, avobenzone, homosalate and padimate-O resulting in a classification of "non-binder" for all four test substances. The mean specific binding for avobenzone, homosalate and padimate-O at 10^{-4} M and 10^{-3} M was not assessed because precipitation was observed at these concentrations. The weak positive control dexamethasone had a $LogIC_{50}$ of -4.5 M while the $LogIC_{50}$ of R1881 was -9.9 M.

In the second valid independent run (14-February-2013), the mean specific binding was > 75% at every soluble concentration tested for avobenzone, ensulizole and homosalate resulting in a classification of "non-binder" for these three test substances. The mean specific binding for avobenzone, homosalate and padimate-O at 10^{-4} M and 10^{-3} M was not assessed because precipitation was observed at these concentrations. The mean specific binding for padimate-O was > 75% at every soluble concentration tested except for 10^{-8} M (44.8%) and 10^{-6} M (75.0%). A Dixon-Q outlier test can eliminate one of the three replicates in the 10^{-6} M data set, resulting in a mean specific binding value of 102.4%. Additionally, the replicates at 10^{-8} M are substantially varied, exhibiting specific binding of -0.3% (replicate 1), 54.6% (replicate 2) and 80.0% (replicate 3). Because all three replicates are so varied, the Dixon-Q outlier test will not allow removal of one or more of the replicates in the analysis. However, these inconsistencies between replicates suggest a loss of all or a part of the HAP pellet during the final extraction and washing steps. Since all soluble concentrations, both higher and lower than 10^{-8} M, exhibit mean specific binding of > 75%, padimate-O is also classified as a "non-binder" for this run. The weak positive control dexamethasone had a $LogIC_{50}$ of -4.5 M while the $LogIC_{50}$ of R1881 was -9.9 M.
Finally, in the third valid independent run (16-February-2013), the mean specific binding was > 75% at every soluble concentration tested for ensulizole, avobenzone, homosalate, and padimate-O, resulting in the classification as a "non-binder" for all four test substances. The mean specific binding for avobenzone, homosalate and padimate-O at 10^{-4} M and 10^{-3} M was not assessed because precipitation was observed at these concentrations. The weak positive control dexamethasone had a $LogIC_{50}$ of -4.6 M while the $LogIC_{50}$ of R1881 was -10.0 M.

The mean relative binding affinity, or RBA (calculated by dividing the $LogIC_{50}$ of the control/test material by the $LogIC_{50}$ of the positive control R1881) was 0.5 for dexamethasone. As ensulizole, avobenzone, homosalate and padimate-O were not classified as an overall "binder" (mean specific binding $\geq$ 50%), the RBA could not be calculated.

5.0 CONCLUSIONS

All four test materials, ensulizole, avobenzone, homosalate and padimate-O are classified as "non-binders" of the AR in all three independent runs and thus have a final classification of "non-binder."

6.0 REFERENCES

Endocrine Disruptor Screening Program Test Guidelines. *OPPTS 890.1150: Androgen Receptor Binding (Rat Prostate Cytosol).* EPA 640-C-09-003. October, 2009.

TABLES SECTION

TABLE 1 Results of 1st Valid Binding Assay – Controls – February 11, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
R1881 (NSB)	-6	0.0	0.6	0.3	2.4E+17
	-7	0.2	0.6	0.4	316.0
	-8	1.4	0.5	0.3	32.5
	-9	10.7	0.9	0.5	8.1
	-10	52.1	4.7	2.7	8.9
	-11	87.8	4.0	2.3	4.6
Dexamethasone	-3	3.1	1.5	0.8	46.7
	-4	18.8	0.9	0.5	4.6
	-5	77.7	9.4	5.4	12.1
	-6	92.4	2.9	1.7	3.2
	-7	95.8	2.1	1.2	2.2
	-8	98.3	2.3	1.3	2.3
	-9	96.7	0.4	0.2	0.4
	-10	95.2	2.6	1.5	2.7

TABLE 2 Results of 1st Valid Binding Assay – Test Articles – February 11, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Ensulizole	-3	94.4	0.9	0.5	0.9
	-4	97.1	2.0	1.2	2.1
	-5	99.8	0.6	0.4	0.6
	-6	97.6	3.7	2.2	3.8
	-7	97.6	1.8	1.1	1.9
	-8	100.9	0.5	0.3	0.5
	-9	99.3	2.9	1.7	2.9
	-10	86.6	20.2	11.6	23.3
Avobenzone	-3	101.3	3.2	1.9	3.2
	-4	68.0	48.8	28.2	71.8
	-5	87.8	12.1	7.0	13.8
	-6	97.5	3.1	1.8	3.1
	-7	96.0	0.8	0.4	0.8
	-8	96.5	7.4	4.3	7.6
	-9	97.5	2.3	1.4	2.4
	-10	96.3	1.5	0.9	1.6
Homosalate	-3	37.2	1.5	0.9	4.0
	-4	51.9	3.2	1.8	6.1
	-5	89.5	1.2	0.7	1.3
	-6	96.6	3.0	1.8	3.2
	-7	96.1	2.8	1.6	3.0
	-8	98.5	1.0	0.6	1.0
	-9	98.6	2.6	1.5	2.7
	-10	99.5	1.3	0.8	1.3
Padimate-O	-3	91.0	2.4	1.4	2.6
	-4	91.4	5.9	3.4	6.4
	-5	98.3	1.6	0.9	1.6
	-6	96.9	3.6	2.1	3.7
	-7	98.5	1.2	0.7	1.2
	-8	98.4	1.7	1.0	1.8
	-9	97.0	2.0	1.1	2.1
	-10	99.8	2.0	1.2	2.0

Red lettering indicates where significant precipitation of test material was observed.

TABLE 3 1st Valid Run - Upper and Lower Parameters in Competitive Assay Binding Curves for the Standards – February 11, 2013

Parameter	Unit	R1881	Dexamethasone
Bottom Plateau Level	% binding	0	2
Top Plateau Level	% binding	95	96
Hill Slope	$Log_{10}(M)^{-1}$	-1.0	-1.3

TABLE 4 Results of 2nd Valid Binding Assay – Controls – February 14, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
R1881 (NSB)	-6	0.0	0.6	0.2	-1.3E+18
	-7	1.1	0.4	0.2	38.9
	-8	2.2	0.7	0.4	30.6
	-9	11.5	0.5	0.3	4.1
	-10	54.4	2.6	1.5	4.7
	-11	93.8	3.6	2.1	3.8
Dexamethasone	-3	2.1	1.7	1.0	80.7
	-4	23.2	0.3	0.2	1.1
	-5	75.0	3.8	2.2	5.1
	-6	92.4	0.8	0.6	0.9
	-7	99.4	2.5	1.4	2.5
	-8	99.6	3.6	2.1	3.6
	-9	105.4	1.5	0.9	1.4
	-10	101.1	1.4	0.8	1.4

TABLE 5 Results of 2nd Valid Binding Assay – Test Articles – February 14, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Ensulizole	-3	84.4	2.1	1.2	2.5
	-4	98.4	0.7	0.4	0.7
	-5	101.9	1.5	0.9	1.5
	-6	103.3	3.6	2.1	3.5
	-7	100.4	2.6	1.5	2.5
	-8	98.9	0.8	0.4	0.8
	-9	98.0	1.1	0.6	1.1
	-10	104.8	1.0	0.6	1.0
Avobenzone	-3	98.0	0.6	0.3	0.6
	-4	98.5	0.6	0.4	0.6
	-5	93.9	6.1	3.5	6.5
	-6	97.4	1.5	0.9	1.6
	-7	99.8	3.4	2.0	3.4
	-8	99.9	1.2	0.7	1.2
	-9	99.1	0.9	0.5	0.9
	-10	94.9	1.6	0.9	1.7
Homosalate	-3	35.2	3.7	2.1	10.5
	-4	46.1	3.5	2.0	7.6
	-5	86.2	2.3	1.3	2.6
	-6	77.0	2.3	1.3	3.0
	-7	103.3	2.0	1.2	2.0
	-8	101.1	1.5	0.9	1.5
	-9	100.9	5.3	3.0	5.2
	-10	93.3	5.6	3.2	6.0
Padimate-O	-3	72.6	5.1	3.0	7.1
	-4	95.4	3.2	1.8	3.3
	-5	103.4	0.7	0.4	0.7
	-6	75.0	47.5	27.4	63.4
	-7	90.0	4.5	2.6	5.0
	-8	44.8	41.0	23.7	91.6
	-9	102.1	5.9	3.4	5.8
	-10	104.1	1.0	0.6	1.0

Red lettering indicates where significant precipitation of test material was observed.

TABLE 6 Results of 2nd Valid Binding Assay - Upper and Lower Parameters in Competitive Assay Binding Curves for the Standards – February 14, 2013

Parameter	Unit	R1881	Dexamethasone
Bottom Plateau Level	% binding	0	-3
Top Plateau Level	% binding	103	101
Hill Slope	$Log_{10}(M)^{-1}$	-1.0	-0.9

TABLE 7 Results of 3rd Valid Binding Assay – Controls – February 16, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
R1881 (NSB)	-6	0.0	1.3	0.6	-1.8E+18
	-7	0.0	0.2	0.1	-526.8
	-8	1.6	0.7	0.4	46.0
	-9	9.5	0.4	0.2	4.3
	-10	52.4	3.6	2.1	6.8
	-11	91.9	1.7	1.0	1.8
Dexamethasone	-3	4.4	2.2	1.3	49.1
	-4	20.6	3.0	1.7	14.4
	-5	73.4	1.8	1.0	2.4
	-6	96.2	1.5	0.9	1.6
	-7	100.6	2.2	1.3	2.2
	-8	102.5	1.8	1.1	1.8
	-9	101.7	2.9	1.7	2.8
	-10	99.2	2.5	1.4	2.5

TABLE 8 Results of 3rd Valid Binding Assay – Test Articles – February 16, 2013

Test Material	Concentration (Log[M])	Specific Binding (%)	Standard Deviation	Standard Error of Mean	% Coefficient of Variation
Ensulizole	-3	93.6	5.6	3.2	6.0
	-4	92.9	6.3	3.6	6.7
	-5	102.6	1.3	0.7	1.3
	-6	101.5	1.5	0.9	1.5
	-7	75.5	43.7	25.2	57.9
	-8	95.3	5.8	3.4	6.1
	-9	100.5	0.6	0.4	0.6
	-10	96.9	2.4	1.4	2.5
Avobenzone	-3	97.0	2.8	1.6	2.9
	-4	94.9	3.5	2.0	3.7
	-5	103.0	3.1	1.8	3.0
	-6	98.7	1.9	1.1	1.9
	-7	95.8	1.2	0.7	1.3
	-8	100.9	0.8	0.5	0.8
	-9	104.1	0.8	0.5	0.8
	-10	90.1	10.2	5.9	11.4
Homosalate	-3	28.2	6.5	3.8	23.1
	-4	57.0	9.1	5.3	16.0
	-5	92.9	3.8	2.2	4.0
	-6	83.6	20.6	11.9	24.6
	-7	99.6	3.4	2.0	3.4
	-8	99.4	1.1	0.6	1.1
	-9	103.0	2.1	1.2	2.0
	-10	99.7	1.2	0.7	1.2
Padimate-O	-3	81.7	10.9	6.3	13.3
	-4	92.7	1.5	0.9	1.6
	-5	99.1	3.2	1.8	3.2
	-6	103.4	2.3	1.3	2.2
	-7	100.0	0.7	0.4	0.7
	-8	91.2	11.5	6.7	12.6
	-9	97.0	0.6	0.4	0.7
	-10	92.3	9.2	5.3	9.9

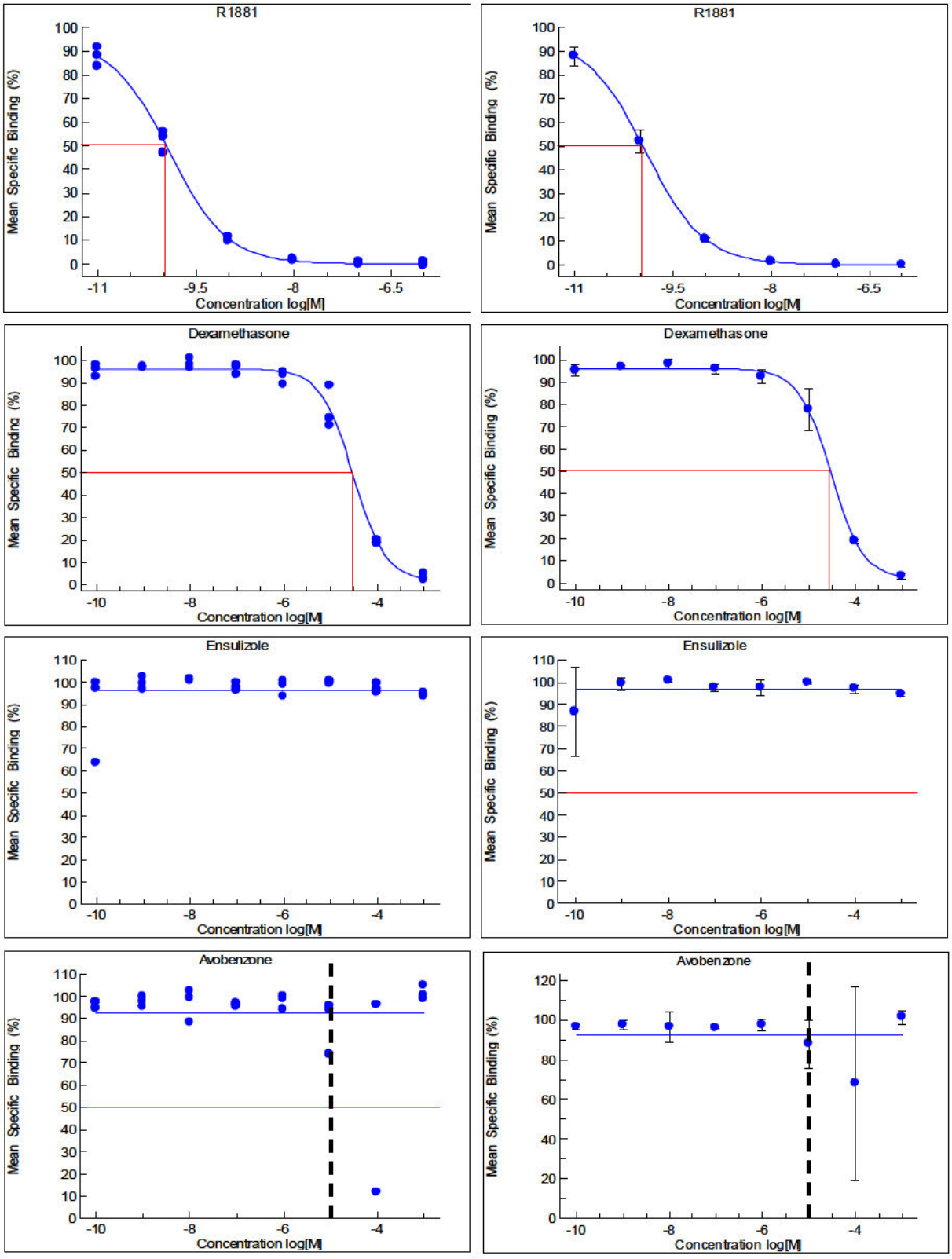
Red lettering indicates where significant precipitation of test material was observed.

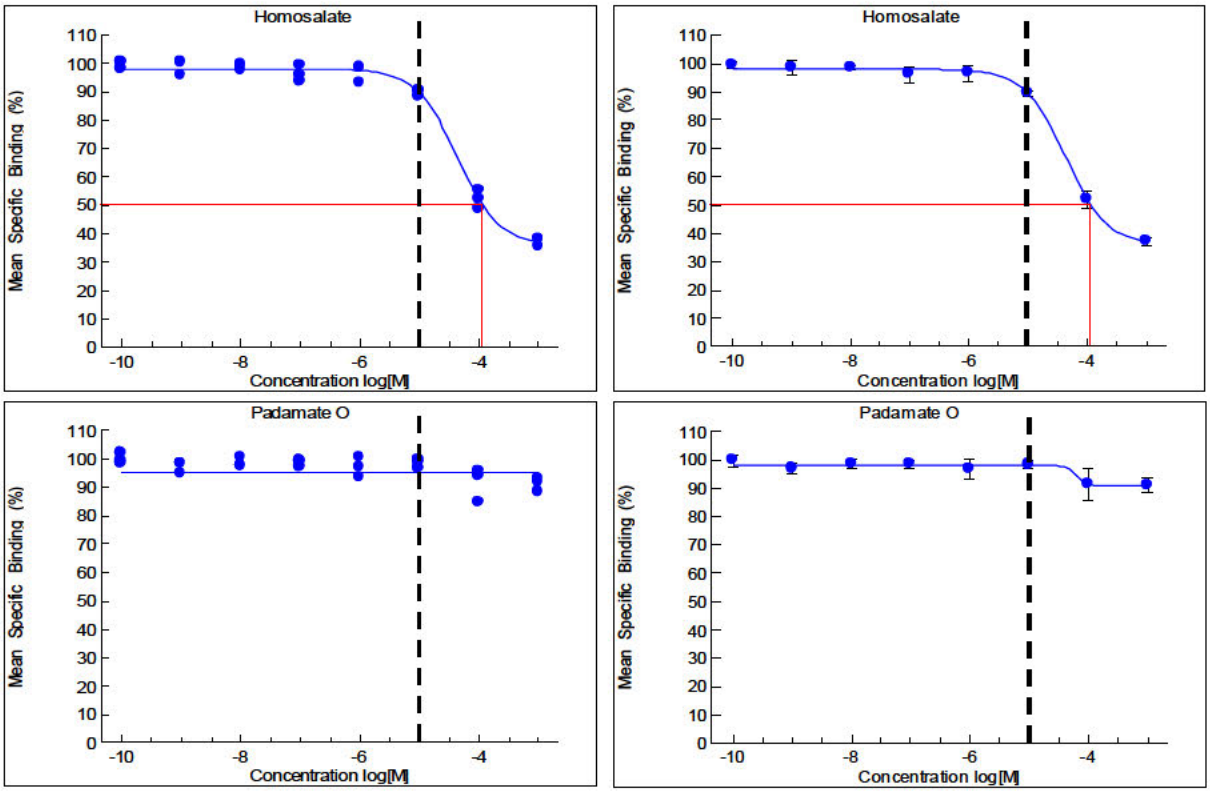
TABLE 9 Results of 3rd Valid Binding Assay - Upper and Lower Parameters in Competitive Assay Binding Curves for the Standards – February 16, 2013

Parameter	Unit	R1881	Dexamethasone
Bottom Plateau Level	% binding	0	2
Top Plateau Level	% binding	100	101
Hill Slope	$Log_{10}(M)^{-1}$	-1.0	-1.0

FIGURES SECTION

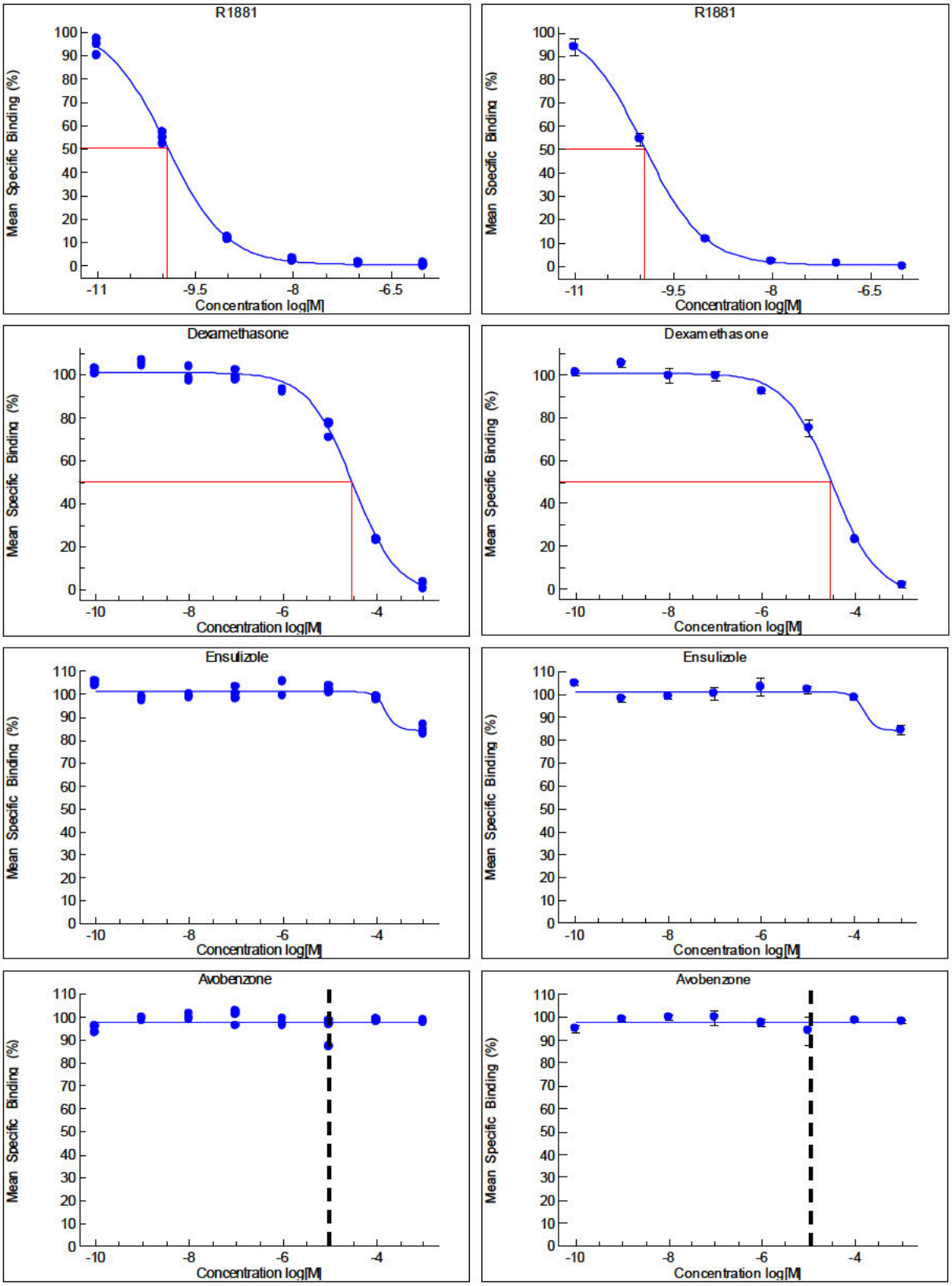
FIGURE 1 1st Valid Run % Specific Binding for Test Substances and Controls – February 11, 2013

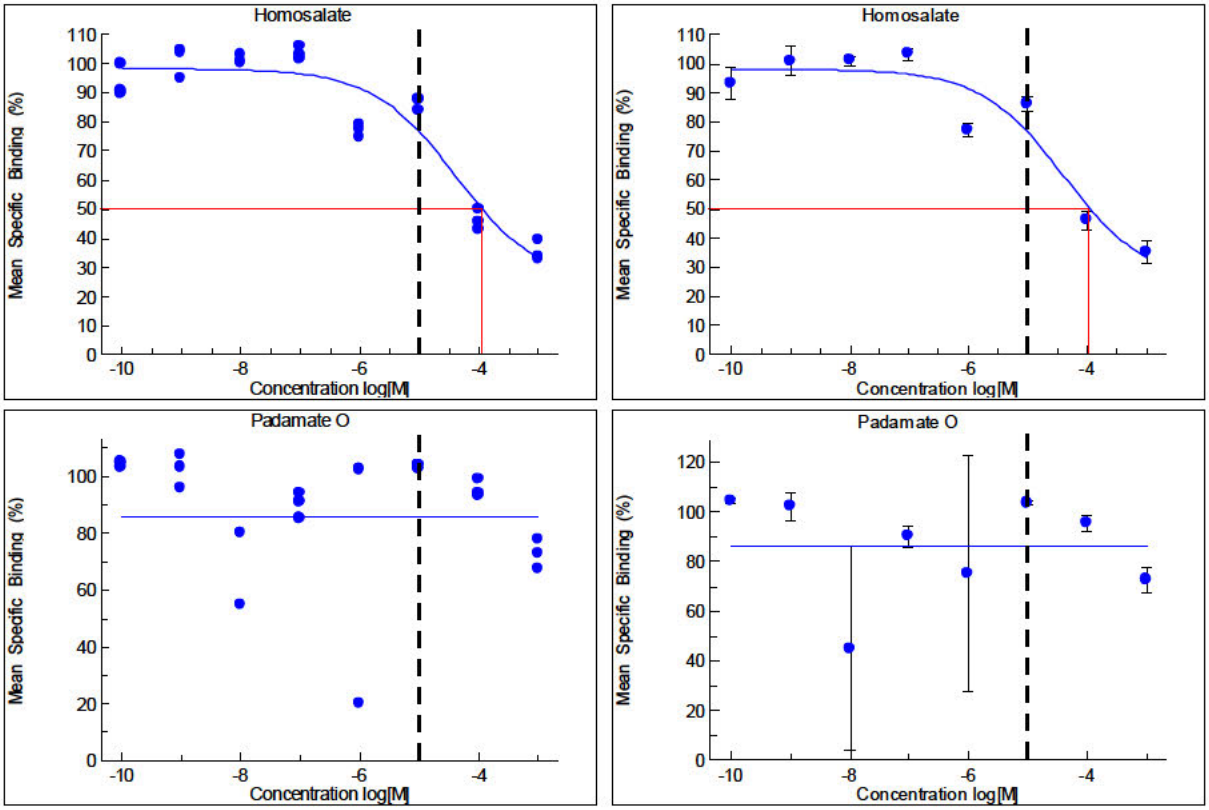




The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the first independent run of the assay (n=3). The vertical black dotted lined represents the limit of solubility as tested in this system. Any concentration at or below this line was considered soluble.

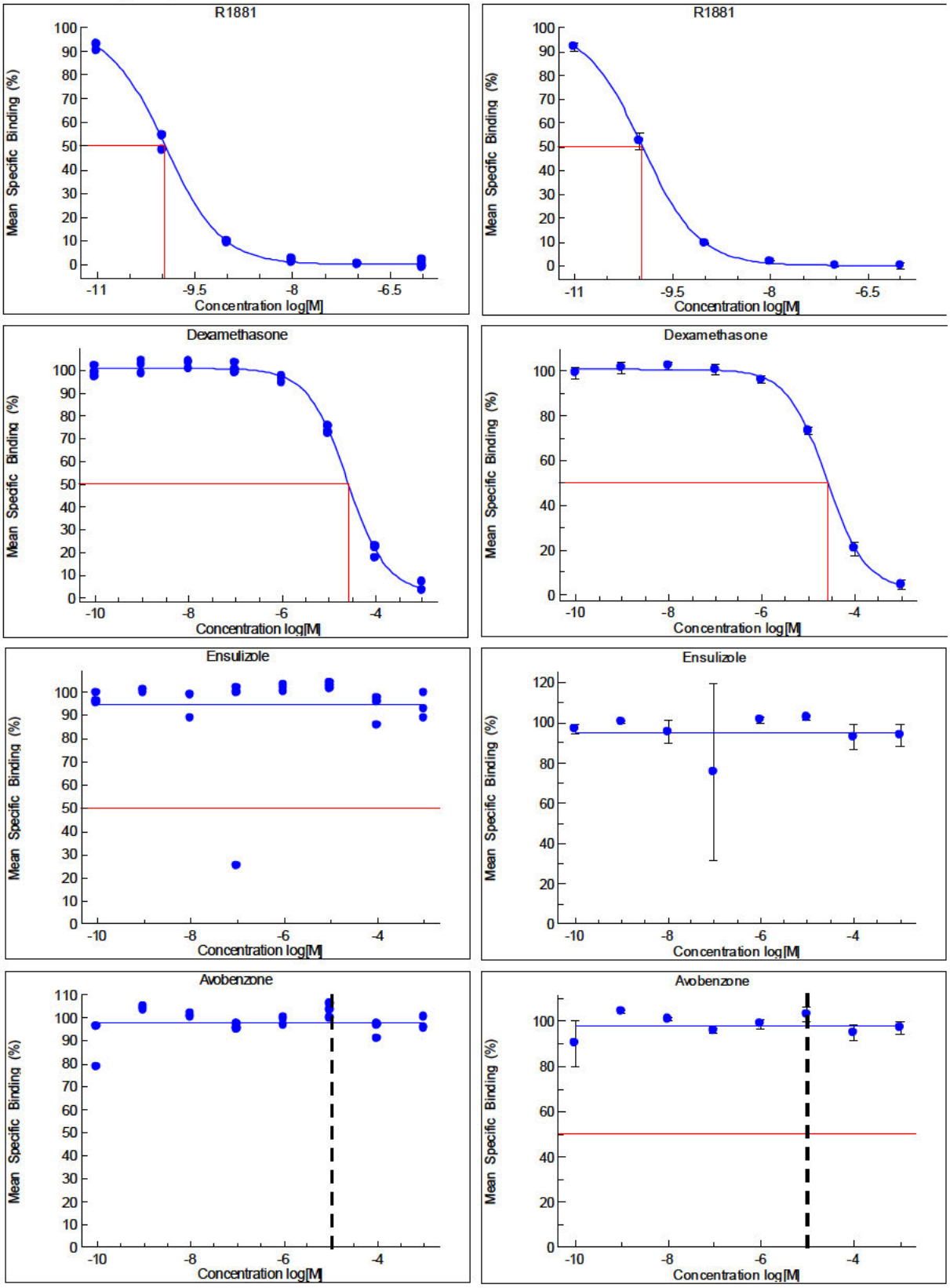
FIGURE 2 2nd Valid Run % Specific Binding for Test Substances and Controls – February 14, 2013

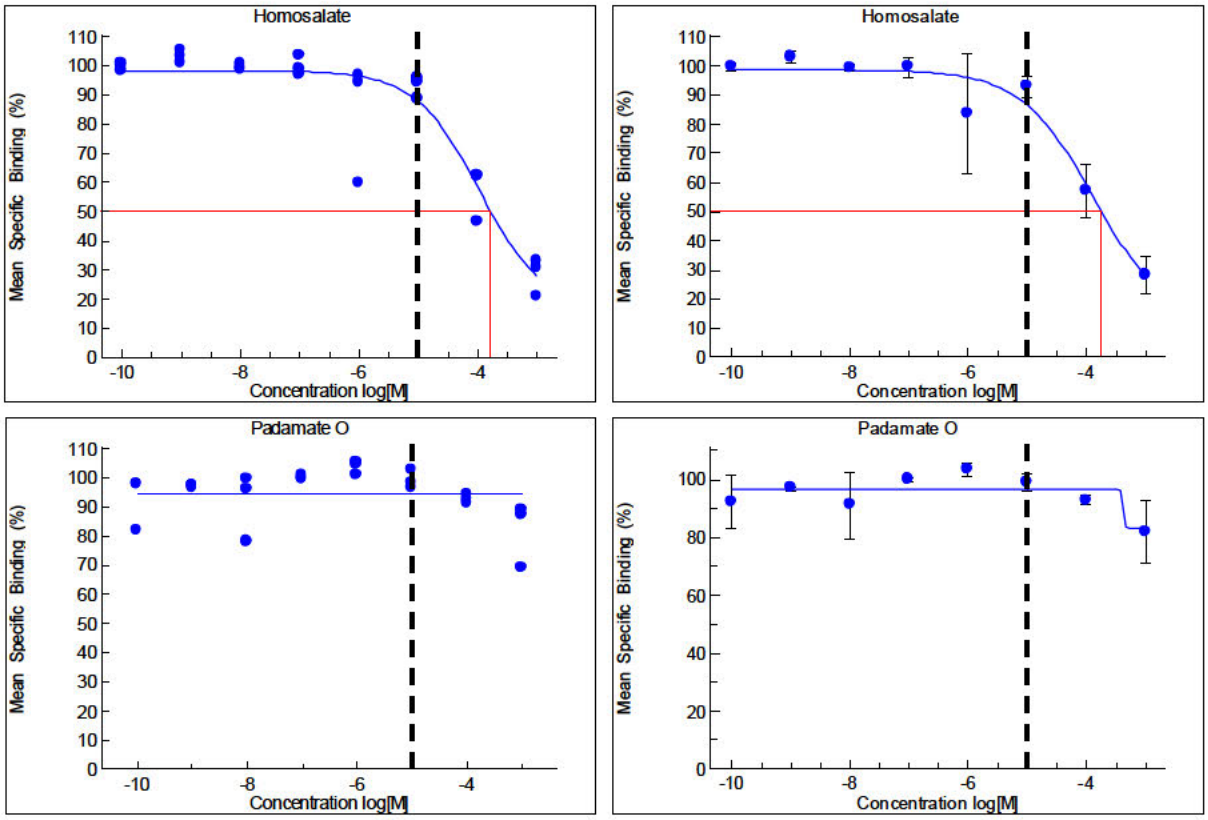




The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the second independent run of the assay (n=3). The vertical black dotted lined represents the limit of solubility as tested in this system. Any concentration at or below this line was considered soluble.

FIGURE 3 3rd Valid Run % Specific Binding for Controls and Test Substances – February 16, 2013





The graphs on the left show individual replicates while graphs on the right show mean data (Means±Standard Deviation) from the third independent run of the assay (n=3). The vertical black dotted lined represents the limit of solubility as tested in this system. Any concentration at or below this line was considered soluble.

APPENDICES SECTION

APPENDIX 1 Raw and Normalized Data Valid Run 1 – February 11, 2013

Experiment Date: 11-Feb-13 Study Number: 9070-100794ARB Assays Conducted by: [redacted]
Test substance: Ensulizole
3/6/2013 15:22

ug protein/assay tube = 270.0

Tube	Sample Type	DPM (1mL)	Specific Binding DPM (100 uL) - NSB	Total Specific Binding (300 uL)	Mean
1	Total Activity (Master Mix)	55960	--	55960.0	55652.7
2		55938	--	55938.0	
3		55142	--	55142.0	
4		55852	--	55852.0	
5		55995	--	55995.0	
6		55229	--	55229.0	
7	Total Binding (Solvent Control)	1786	1687.2	5062	6177.6
8		1686	1787.2	5362	
9		1621	1722.2	5167	
10		1811	1712.2	5137	
11		1871	1772.2	5317	
12		1773	1674.2	5023	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (3mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
95.0	13	R1881 (NSB)	-6	-3.8	-11.5	-0.2	-0.3	0.1	0.0	0.6	0.3	2.4E+17	0.5
89.0	14		-6	-9.8	-29.5	-0.6	-0.6	0.4					0.5
98.0	15		-6	-0.8	-2.5	0.0	-0.1	0.0					0.5
112.0	16		-6	13.2	39.5	0.8	0.7	0.5					0.6
112.0	17		-6	13.2	39.5	0.8	0.7	0.5					0.6
87.0	18		-6	-11.8	-35.5	-0.7	-0.7	0.6					0.5
92.0	19	R1881	-7	-6.8	-20.5	-0.4	-0.6	0.3	0.2	0.6	0.4	316.0	0.5
114.0	20		-7	15.2	45.5	0.9	0.7	0.5					0.6
101.0	21		-7	2.2	6.5	0.1	-0.1	0.0					0.5
133.0	22	R1881	-8	34.2	102.5	2.0	0.7	0.5	1.4	0.5	0.3	32.5	0.7
119.0	23		-8	20.2	60.5	1.2	-0.1	0.0					0.6
119.0	24		-8	20.2	60.5	1.2	-0.1	0.0					0.6
266.0	25	R1881	-9	167.2	501.5	9.7	-1.0	1.1	10.7	0.9	0.5	8.1	1.4
292.0	26		-9	193.2	579.5	11.2	0.5	0.2					1.6
292.0	27		-9	193.2	579.5	11.2	0.5	0.2					1.6
1061.0	28	R1881	-10	962.2	2886.5	55.8	3.7	13.7	52.1	4.7	2.7	8.9	5.7
907.0	29		-10	808.2	2424.5	46.8	-5.2	27.3					4.9
1024.0	30		-10	925.2	2775.5	53.6	1.6	2.4					5.5
1621.0	31	R1881	-11	1522.2	4566.5	88.2	0.4	0.2	87.8	4.0	2.3	4.6	8.7
1541.0	32		-11	1442.2	4326.5	83.6	-4.2	17.8					8.3
1679.0	33		-11	1580.2	4740.5	91.6	3.8	14.3					9.1
144.0	37	Dexamethasone	-3	45.2	135.5	2.6	-0.4	0.1	3.1	1.5	0.8	46.7	0.8
133.0	38		-3	34.2	102.5	2.0	-1.0	1.0					0.7
181.0	39		-3	82.2	246.5	4.8	1.8	3.2					1.0
419.0	40	Dexamethasone	-4	320.2	960.5	18.6	-0.5	0.2	18.8	0.9	0.5	4.6	2.3
440.0	41		-4	341.2	1023.5	19.8	0.7	0.5					2.4
411.0	42		-4	312.2	936.5	18.1	-0.9	0.9					2.2
1375.0	43	Dexamethasone	-5	1276.2	3828.5	73.9	-3.4	11.3	77.7	9.4	5.4	12.1	7.4
1320.0	44		-5	1221.2	3663.5	70.8	-6.5	42.6					7.1
1625.0	45		-5	1526.2	4578.5	88.4	11.1	123.8					8.8
1636.0	46	Dexamethasone	-6	1537.2	4611.5	89.1	-5.7	32.1	92.4	2.9	1.7	3.2	8.8
1732.0	47		-6	1633.2	4899.5	94.6	-0.1	0.0					9.3
1711.0	48		-6	1612.2	4836.5	93.4	-1.3	1.7					9.2
1711.0	49	Dexamethasone	-7	1612.2	4836.5	93.4	-1.3	1.7	95.8	2.1	1.2	2.2	9.2
1781.0	50		-7	1682.2	5046.5	97.6	1.5	2.3					9.6
1766.0	51		-7	1667.2	5001.5	96.6	0.7	0.4					9.5
1784.0	52	Dexamethasone	-8	1685.2	5055.5	97.6	1.6	2.7	96.3	2.3	1.3	2.3	9.6
1762.0	53		-8	1663.2	4989.5	96.4	0.4	0.1					9.5
1839.0	54		-8	1740.2	5220.5	100.8	4.8	23.3					9.9
1769.0	55	Dexamethasone	-9	1670.2	5010.5	96.8	0.8	0.6	96.7	0.4	0.2	0.4	9.5
1759.0	56		-9	1660.2	4980.5	96.2	0.2	0.0					9.5
1773.0	57		-9	1674.2	5022.5	97.0	1.0	1.0					9.6
1754.0	58	Dexamethasone	-10	1655.2	4965.5	95.9	-0.1	0.0	95.2	2.6	1.5	2.7	9.5
1693.0	59		-10	1594.2	4782.5	92.4	-3.6	13.3					9.1
1781.0	60		-10	1682.2	5046.5	97.5	1.5	2.3					9.6
1715.0	61	Ensulizole	-3	1616.2	4848.5	93.6	-3.0	9.2	94.4	0.9	0.5	0.9	9.2
1744.0	62		-3	1645.2	4935.5	95.3	-1.3	1.8					9.4
1724.0	63		-3	1625.2	4875.5	94.2	-2.5	6.3					9.3
1745.0	64	Ensulizole	-4	1646.2	4938.5	95.4	-1.3	1.7	97.1	2.0	1.2	2.1	9.4
1767.0	65		-4	1668.2	5004.5	96.7	0.0	0.0					9.5
1814.0	66		-4	1715.2	5145.5	99.4	2.7	7.3					9.8
1826.0	67	Ensulizole	-5	1727.2	5181.5	100.1	3.4	11.6	99.8	0.6	0.4	0.6	9.8
1810.0	68		-5	1711.2	5133.5	99.2	2.5	6.1					9.8
1830.0	69		-5	1731.2	5193.5	100.3	3.6	13.2					9.9
1710.0	70	Ensulizole	-6	1611.2	4833.5	93.4	-3.3	11.0	97.6	3.7	2.2	3.8	9.2
1806.0	71		-6	1707.2	5121.5	98.9	2.2	5.0					9.7
1833.0	72		-6	1734.2	5202.5	100.5	3.8	14.5					9.9
1768.0	73	Ensulizole	-7	1657.2	4971.5	96.0	-0.7	0.4	97.6	1.8	1.1	1.9	9.5
1777.0	74		-7	1678.2	5034.5	97.2	0.6	0.3					9.6
1819.0	75		-7	1719.2	5157.5	99.6	2.9	8.6					9.8
1841.0	76	Ensulizole	-8	1742.2	5226.5	100.9	4.3	18.3	100.9	0.5	0.3	0.5	9.9
1831.0	77		-8	1732.2	5196.5	100.4	3.7	13.6					9.9
1849.0	78		-8	1750.2	5250.5	101.4	4.7	22.4					10.0
1808.0	79	Ensulizole	-9	1709.2	5127.5	99.0	2.4	5.6	99.3	2.9	1.7	2.9	9.7
1864.0	80		-9	1765.2	5295.5	102.3	5.6	31.4					10.0
1764.0	81		-9	1665.2	4995.5	96.5	-0.2	0.0					9.5
1819.0	82	Ensulizole	-10	1719.2	5157.5	99.6	2.9	8.6	86.6	20.2	11.6	23.3	9.8
1771.0	83		-10	1672.2	5016.5	96.9	0.2	0.0					9.5
1193.0	84		-10	1094.2	3282.5	63.4	-33.3	1107.1					6.4

1909.0	61	Avobenzone	-3	1810.2	5430.5	104.9	12.3	150.8	101.3	3.2	1.9	3.2	10.3
1834.0	62		-3	1735.2	5205.5	100.5	7.9	62.9					9.9
1800.0	63		-3	1701.2	5103.5	98.6	6.0	35.6					9.7
1759.0	64	Avobenzone	-4	1660.2	4980.5	96.2	3.6	12.9	68.0	48.8	28.2	71.8	9.5
1759.0	65		-4	1660.2	4980.5	96.2	3.6	12.9					9.5
299.0	66		-4	200.2	600.5	11.6	-81.0	6562.5					1.6
1373.0	67	Avobenzone	-5	1274.2	3822.5	73.8	-18.8	352.6	87.8	12.1	7.0	13.8	7.4
1719.0	68		-5	1620.2	4860.5	93.9	1.3	1.6					9.3
1748.0	69		-5	1649.2	4947.5	95.6	3.0	8.7					9.4
1823.0	70	Avobenzone	-6	1724.2	5172.5	99.9	7.3	53.2	97.5	3.1	1.8	3.1	9.8
1722.0	71		-6	1623.2	4869.5	94.1	1.4	2.1					9.3
1800.0	72		-6	1701.2	5103.5	98.6	6.0	35.6					9.7
1759.0	73	Avobenzone	-7	1660.2	4980.5	96.2	3.6	12.9	96.0	0.8	0.4	0.8	9.5
1767.0	74		-7	1668.2	5004.5	96.7	4.1	16.4					9.5
1741.0	75		-7	1642.2	4926.5	95.2	2.5	6.5					9.4
1864.0	76	Avobenzone	-8	1765.2	5295.5	102.3	9.7	93.5	96.5	7.4	4.3	7.6	10.0
1621.0	77		-8	1522.2	4566.5	88.2	-4.4	19.4					8.7
1807.0	78		-8	1708.2	5124.5	99.0	6.4	40.6					9.7
1740.0	79	Avobenzone	-9	1641.2	4923.5	95.1	2.5	6.2	97.5	2.3	1.4	2.4	9.4
1783.0	80		-9	1684.2	5052.5	97.6	5.0	24.8					9.6
1821.0	81		-9	1722.2	5166.5	99.8	7.2	51.6					9.8
1778.0	82	Avobenzone	-10	1679.2	5037.5	97.3	4.7	22.0	96.3	1.5	0.9	1.6	9.6
1730.0	83		-10	1631.2	4893.5	94.5	1.9	3.6					9.3
1774.0	84		-10	1675.2	5025.5	97.1	4.5	19.9					9.6
711.0	61	Homosalate	-3	612.2	1836.5	35.5	-1.7	2.8	37.2	1.5	0.9	4.0	3.8
754.0	62		-3	655.2	1965.5	38.0	0.8	0.7					4.1
756.0	63		-3	657.2	1971.5	38.1	0.9	0.9					4.1
996.0	64	Homosalate	-4	897.2	2691.5	52.0	0.0	0.0	51.9	3.2	1.8	6.1	5.4
939.0	65		-4	840.2	2520.5	48.7	-3.3	10.8					5.1
1049.0	66		-4	950.2	2850.5	55.1	3.1	9.6					5.7
1650.0	67	Homosalate	-5	1551.2	4653.5	89.9	0.5	0.3	89.5	1.2	0.7	1.3	8.9
1621.0	68		-5	1522.2	4566.5	88.2	-1.1	1.3					8.7
1659.0	69		-5	1560.2	4680.5	90.4	1.1	1.1					8.9
1798.0	70	Homosalate	-6	1699.2	5097.5	98.5	1.0	1.0	96.6	3.0	1.8	3.2	9.7
1705.0	71		-6	1606.2	4818.5	93.1	-4.4	19.2					9.2
1794.0	72		-6	1695.2	5085.5	98.2	0.8	0.6					9.7
1713.0	73	Homosalate	-7	1614.2	4842.5	93.5	-4.5	19.8	96.1	2.8	1.6	3.0	9.2
1750.0	74		-7	1651.2	4953.5	95.7	-2.3	5.3					9.4
1810.0	75		-7	1711.2	5133.5	99.2	1.2	1.4					9.8
1816.0	76	Homosalate	-8	1717.2	5151.5	99.5	1.5	2.2	98.5	1.0	0.6	1.0	9.8
1781.0	77		-8	1682.2	5046.5	97.5	-0.5	0.3					9.6
1801.0	78		-8	1702.2	5106.5	98.6	0.6	0.4					9.7
1830.0	79	Homosalate	-9	1731.2	5193.5	100.3	2.3	5.3	98.6	2.6	1.5	2.7	9.9
1823.0	80		-9	1724.2	5172.5	99.9	1.9	3.6					9.8
1748.0	81		-9	1649.2	4947.5	95.6	-2.5	6.0					9.4
1823.0	82	Homosalate	-10	1724.2	5172.5	99.9	1.9	3.6	99.5	1.3	0.8	1.3	9.8
1791.0	83		-10	1692.2	5076.5	98.0	0.0	0.0					9.7
1835.0	84		-10	1736.2	5208.5	100.6	2.6	6.7					9.9
1623.0	61	Padamate O	-3	1524.2	4572.5	88.3	-7.0	48.4	91.0	2.4	1.4	2.6	8.7
1680.0	62		-3	1581.2	4743.5	91.6	-3.7	13.4					9.1
1704.0	63		-3	1605.2	4815.5	93.0	-2.3	5.1					9.2
1719.0	64	Padamate O	-4	1620.2	4860.5	93.9	-1.4	1.9	91.4	5.9	3.4	6.4	9.3
1561.0	65		-4	1462.2	4386.5	84.7	-10.6	111.3					8.4
1751.0	66		-4	1652.2	4956.5	95.7	0.5	0.2					9.4
1805.0	67	Padamate O	-5	1706.2	5118.5	98.9	3.6	12.9	98.3	1.6	0.9	1.6	9.7
1816.0	68		-5	1717.2	5151.5	99.5	4.2	17.8					9.8
1765.0	69		-5	1666.2	4998.5	96.5	1.3	1.6					9.5
1709.0	70	Padamate O	-6	1610.2	4830.5	93.3	-2.0	3.9	96.9	3.6	2.1	3.7	9.2
1770.0	71		-6	1671.2	5013.5	96.8	1.6	2.4					9.5
1832.0	72		-6	1733.2	5199.5	100.4	5.2	26.5					9.9
1806.0	73	Padamate O	-7	1707.2	5121.5	98.9	3.6	13.3	98.5	1.2	0.7	1.2	9.7
1814.0	74		-7	1715.2	5145.5	99.4	4.1	16.9					9.8
1775.0	75		-7	1676.2	5028.5	97.1	1.8	3.4					9.6
1832.0	76	Padamate O	-8	1733.2	5199.5	100.4	5.2	26.5	98.4	1.7	1.0	1.8	9.9
1783.0	77		-8	1684.2	5052.5	97.6	2.3	5.3					9.6
1777.0	78		-8	1678.2	5034.5	97.2	2.0	3.9					9.6
1793.0	79	Padamate O	-9	1694.2	5082.5	98.2	2.9	8.4	97.0	2.0	1.1	2.1	9.7
1792.0	80		-9	1693.2	5079.5	98.1	2.8	8.0					9.7
1733.0	81		-9	1634.2	4902.5	94.7	-0.6	0.3					9.3
1859.0	82	Padamate O	-10	1760.2	5280.5	102.0	6.7	45.1	99.8	2.0	1.2	2.0	10.0
1811.0	83		-10	1712.2	5136.5	99.2	3.9	15.5					9.8
1792.0	84		-10	1693.2	5079.5	98.1	2.8	8.0					9.7

APPENDIX 1 Raw and Normalized Data Valid Run 2 – February 14, 2013

Experiment Date: 14-Feb-13 Study Number: 9070-100794ARB Assays Conducted by: [redacted]
Test substance: Ensulizole
3/6/2013 15:22

ug protein/assay tube = 284.2

Tube	Sample Type	DPM (1mL)	Specific Binding DPM (100 uL) - NSB	Total Specific Binding (300 uL)	Mean
1	Total Activity (Master Mix)	56285	--	56285.0	53642.0
2		46090	--	46090.0	
3		55216	--	55216.0	
4		55893	--	55893.0	
5		55238	--	55238.0	
6		51121	--	51121.0	
7	Total Binding (Solvent Control)	1603	1495.0	4485	4616.6
8		1667	1559.0	4677	
9		1678	1570.0	4710	
10		1579	1471.0	4413	
11		1694	1586.0	4758	
12		1664	1556.0	4668	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (3mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
108.0	13	R1881 (NSB)	-6	-2.0	-6.0	-0.1	-0.5	0.3	0.0	0.6	0.2	-1.3E+16	0.6
105.0	14		-6	-3.0	-9.0	-0.2	-0.6	0.4					0.6
99.0	15		-6	-9.0	-27.0	-0.6	-1.0	1.0					0.6
103.0	16		-6	-5.0	-15.0	-0.3	-0.7	0.5					0.6
125.0	17		-6	17.0	51.0	1.1	0.7	0.5					0.7
110.0	18		-6	2.0	6.0	0.1	-0.3	0.1					0.6
117.0	19	R1881	-7	9.0	27.0	0.6	0.0	0.0	1.1	0.4	0.2	38.9	0.7
128.0	20		-7	20.0	60.0	1.3	0.8	0.6					0.7
128.0	21		-7	20.0	60.0	1.3	0.8	0.6					0.7
137.0	22	R1881	-8	29.0	87.0	1.9	0.1	0.0	2.2	0.7	0.4	30.6	0.8
134.0	23		-8	26.0	78.0	1.7	-0.1	0.0					0.7
153.0	24		-8	45.0	135.0	2.9	1.2	1.3					0.9
293.0	25	R1881	-9	185.0	555.0	12.0	0.4	0.2	11.5	0.5	0.3	4.1	1.6
281.0	26		-9	173.0	519.0	11.2	-0.4	0.1					1.6
280.0	27		-9	172.0	516.0	11.2	-0.4	0.2					1.6
906.0	28	R1881	-10	798.0	2394.0	51.6	2.5	6.4	54.4	2.6	1.5	4.7	5.1
995.0	29		-10	877.0	2631.0	57.0	2.6	6.8					5.5
945.0	30		-10	837.0	2511.0	54.4	0.0	0.0					5.3
1493.0	31	R1881	-11	1385.0	4155.0	90.0	3.8	14.8	93.8	3.6	2.1	3.8	8.3
1601.0	32		-11	1493.0	4479.0	97.0	3.2	10.1					9.0
1562.0	33		-11	1454.0	4362.0	94.4	0.6	0.4					8.7
148.0	37	Dexamethasone	-3	40.0	120.0	2.6	0.9	0.9	2.1	1.7	1.0	80.7	0.8
160.0	38		-3	52.0	156.0	3.4	1.7	3.0					0.9
111.0	39		-3	3.0	9.0	0.2	-1.5	2.1					0.6
464.0	40	Dexamethasone	-4	356.0	1068.0	23.1	-0.9	0.8	23.2	0.3	0.2	1.1	2.6
461.0	41		-4	353.0	1059.0	22.9	-1.1	1.2					2.6
469.0	42		-4	361.0	1083.0	23.4	-0.8	0.6					2.6
1194.0	43	Dexamethasone	-5	1086.0	3258.0	70.5	-3.0	9.3	75.0	3.8	2.2	5.1	6.7
1302.0	44		-5	1194.0	3582.0	77.6	4.0	15.6					7.3
1290.0	45		-5	1182.0	3546.0	76.8	3.2	10.2					7.2
1522.0	46	Dexamethasone	-6	1414.0	4242.0	91.8	-4.8	22.8	92.4	0.8	0.6	0.9	8.5
1540.0	47		-6	1432.0	4296.0	93.0	-3.6	13.0					8.6
	48		-6										
1607.0	49	Dexamethasone	-7	1499.0	4497.0	97.4	-3.1	9.9	99.4	2.5	1.4	2.5	9.0
1627.0	50		-7	1519.0	4557.0	98.7	-1.8	3.4					9.1
1680.0	51		-7	1572.0	4716.0	102.1	1.6	2.6					9.4
1703.0	52	Dexamethasone	-8	1595.0	4785.0	103.6	2.6	6.7	99.6	3.6	2.1	3.6	9.5
1598.0	53		-8	1490.0	4470.0	96.8	-4.2	17.9					8.9
1624.0	54		-8	1516.0	4548.0	98.5	-2.5	6.5					9.1
1707.0	55	Dexamethasone	-9	1599.0	4797.0	103.9	2.8	7.8	105.4	1.5	0.9	1.4	9.5
1733.0	56		-9	1625.0	4875.0	105.6	4.5	20.0					9.7
1753.0	57		-9	1645.0	4935.0	106.9	5.8	33.3					9.8
1689.0	58	Dexamethasone	-10	1581.0	4743.0	102.7	1.6	2.6	101.1	1.4	0.8	1.4	9.4
1651.0	59		-10	1543.0	4629.0	100.2	-0.9	0.7					9.2
1654.0	60		-10	1546.0	4638.0	100.4	-0.7	0.4					9.3
1375.0	61	Ensulizole	-3	1267.0	3801.0	82.3	-2.1	4.3	84.4	2.1	1.2	2.5	7.7
1405.0	62		-3	1297.0	3891.0	84.2	-0.1	0.0					7.9
1441.0	63		-3	1333.0	3999.0	86.6	2.2	4.9					8.1
1631.0	64	Ensulizole	-4	1523.0	4569.0	98.9	0.5	0.2	98.4	0.7	0.4	0.7	9.1
1611.0	65		-4	1503.0	4509.0	97.6	-0.8	0.6					9.0
1623.0	66		-4	1520.0	4560.0	98.7	0.3	0.1					9.1
1703.0	67	Ensulizole	-5	1595.0	4785.0	103.6	2.4	5.7	101.9	1.5	0.9	1.5	9.5
1656.0	68		-5	1548.0	4644.0	100.6	-0.7	0.4					9.3
1673.0	69		-5	1565.0	4695.0	101.7	0.4	0.2					9.4
1633.0	70	Ensulizole	-6	1525.0	4575.0	99.1	-2.2	4.8	103.3	3.6	2.1	3.5	9.1
1728.0	71		-6	1620.0	4860.0	105.2	4.0	16.2					9.7
1732.0	72		-6	1624.0	4872.0	105.5	4.3	18.3					9.7
1617.0	73	Ensulizole	-7	1509.0	4527.0	98.0	-3.2	10.2	100.4	2.6	1.5	2.5	9.0
1648.0	74		-7	1540.0	4620.0	100.0	-1.2	1.4					9.2
1695.0	75		-7	1587.0	4761.0	103.1	1.9	3.6					9.5
1621.0	76	Ensulizole	-8	1513.0	4539.0	98.3	-2.9	8.6	98.8	0.6	0.4	0.6	9.1
1628.0	77		-8	1520.0	4560.0	98.7	0.3	0.1					9.1
1644.0	78		-8	1536.0	4608.0	99.8	-1.4	2.1					9.2
1631.0	79	Ensulizole	-9	1523.0	4569.0	98.9	0.5	0.2	98.0	1.1	0.6	1.1	9.1
1621.0	80		-9	1513.0	4539.0	98.3	-2.9	8.6					9.1
1598.0	81		-9	1490.0	4470.0	96.8	-4.4	19.6					8.9
1734.0	82	Ensulizole	-10	1626.0	4878.0	105.6	4.4	19.4	104.8	1.0	0.6	1.0	9.7
1703.0	83		-10	1595.0	4785.0	103.6	2.4	6.7					9.5
1725.0	84		-10	1617.0	4851.0	105.0	3.8	14.6					9.6

APPENDIX 1 Raw and Normalized Data Valid Run 2 – February 14, 2013 (continued)

1624.0	61	Avobenzone	-3	1516.0	4548.0	98.5	0.8	0.6	98.0	0.6	0.3	0.6	9.1
1607.0	62		-3	1499.0	4497.0	97.4	-0.3	0.1					9.0
1617.0	63		-3	1509.0	4527.0	98.0	0.3	0.1					9.0
1614.0	64	Avobenzone	-4	1506.0	4518.0	97.8	0.1	0.0	98.5	0.6	0.4	0.6	9.0
1630.0	65		-4	1522.0	4566.0	98.9	1.2	1.4					9.1
1631.0	66		-4	1523.0	4569.0	98.9	1.2	1.5					9.1
1594.0	67	Avobenzone	-5	1486.0	4458.0	96.5	-1.2	1.4	93.9	6.1	3.5	6.5	8.9
1621.0	68		-5	1513.0	4539.0	98.3	0.6	0.3					9.1
1447.0	69		-5	1339.0	4017.0	87.0	-10.7	114.9					8.1
1603.0	70	Avobenzone	-6	1495.0	4485.0	97.1	-0.6	0.3	97.4	1.5	0.9	1.6	9.0
1633.0	71		-6	1525.0	4575.0	99.1	1.4	1.9					9.1
1587.0	72		-6	1479.0	4437.0	96.1	-1.6	2.6					8.9
1586.0	73	Avobenzone	-7	1478.0	4434.0	96.0	-1.7	2.9	99.8	3.4	2.0	3.4	8.9
1685.0	74		-7	1577.0	4731.0	102.4	4.7	22.5					9.4
1664.0	75		-7	1556.0	4668.0	101.1	3.4	11.4					9.3
1643.0	76	Avobenzone	-8	1535.0	4605.0	99.7	2.0	4.0	99.9	1.2	0.7	1.2	9.2
1629.0	77		-8	1521.0	4563.0	98.8	1.1	1.2					9.1
1667.0	78		-8	1559.0	4677.0	101.3	3.6	12.7					9.3
1639.0	79	Avobenzone	-9	1531.0	4593.0	99.4	1.8	3.1	99.1	0.9	0.5	0.9	9.2
1618.0	80		-9	1510.0	4530.0	98.1	0.4	0.1					9.0
1643.0	81		-9	1535.0	4605.0	99.7	2.0	4.0					9.2
1541.0	82	Avobenzone	-10	1433.0	4299.0	93.1	-4.6	21.3	94.9	1.6	0.9	1.7	8.6
1583.0	83		-10	1475.0	4425.0	95.8	-1.9	3.6					8.9
1583.0	84		-10	1475.0	4425.0	95.8	-1.9	3.6					8.9
611.0	61	Homosalate	-3	503.0	1509.0	32.7	-0.7	0.6	35.2	3.7	2.1	10.5	3.4
623.0	62		-3	515.0	1545.0	33.5	0.0	0.0					3.5
715.0	63		-3	607.0	1821.0	39.4	6.0	36.1					4.0
769.0	64	Homosalate	-4	661.0	1983.0	42.9	-8.3	69.1	46.1	3.5	2.0	7.6	4.3
875.0	65		-4	767.0	2301.0	49.8	-1.4	2.0					4.9
807.0	66		-4	699.0	2097.0	45.4	-5.8	34.2					4.5
1452.0	67	Homosalate	-5	1344.0	4032.0	87.3	10.9	118.7	86.2	2.3	1.3	2.6	8.1
1459.0	68		-5	1351.0	4053.0	87.8	11.3	128.8					8.2
1395.0	69		-5	1287.0	3861.0	83.6	7.2	51.7					7.8
1254.0	70	Homosalate	-6	1146.0	3438.0	74.4	-17.0	290.4	77.0	2.3	1.3	3.0	7.0
1325.0	71		-6	1217.0	3651.0	79.1	-12.4	154.5					7.4
1299.0	72		-6	1191.0	3573.0	77.4	-14.1	199.3					7.3
1671.0	73	Homosalate	-7	1563.0	4689.0	101.5	5.1	25.6	103.3	2.0	1.2	2.0	9.3
1692.0	74		-7	1584.0	4752.0	102.9	6.4	41.2					9.5
1733.0	75		-7	1625.0	4875.0	105.6	9.1	82.5					9.7
1691.0	76	Homosalate	-8	1583.0	4749.0	102.8	5.1	25.6	101.1	1.5	0.9	1.5	9.5
1658.0	77		-8	1550.0	4650.0	100.7	2.9	8.5					9.3
1645.0	78		-8	1537.0	4611.0	99.8	2.1	4.3					9.2
1702.0	79	Homosalate	-9	1594.0	4782.0	103.5	5.5	29.7	100.9	5.3	3.0	5.2	9.5
1714.0	80		-9	1606.0	4818.0	104.3	6.2	38.9					9.6
1568.0	81		-9	1460.0	4380.0	94.8	-3.2	10.6					8.8
1487.0	82	Homosalate	-10	1379.0	4137.0	89.6	-8.6	73.7	93.3	5.6	3.2	6.0	8.3
1644.0	83		-10	1536.0	4608.0	99.8	1.6	2.6					9.2
1502.0	84		-10	1394.0	4182.0	90.5	-7.6	58.0					8.4
1145.0	61	Padamate O	-3	1037.0	3111.0	67.4	-18.6	344.9	72.6	5.1	3.0	7.1	6.4
1230.0	62		-3	1122.0	3366.0	72.9	-13.1	170.3					6.9
1303.0	63		-3	1195.0	3585.0	77.6	-8.3	69.0					7.3
1543.0	64	Padamate O	-4	1435.0	4305.0	93.2	7.3	53.0	95.4	3.2	1.8	3.3	8.6
1556.0	65		-4	1448.0	4344.0	94.1	8.1	66.0					8.7
1633.0	66		-4	1525.0	4575.0	99.1	13.1	172.3					9.1
1708.0	67	Padamate O	-5	1600.0	4800.0	103.9	18.0	323.9	103.4	0.7	0.4	0.7	9.6
1688.0	68		-5	1580.0	4740.0	102.6	16.7	278.9					9.4
1705.0	69		-5	1597.0	4791.0	103.7	17.8	317.0					9.5
1683.0	70	Padamate O	-6	1575.0	4725.0	102.3	16.4	268.1	75.0	47.5	27.4	63.4	9.4
1686.0	71		-6	1578.0	4734.0	102.5	16.6	274.5					9.4
417.0	72		-6	309.0	927.0	20.1	-65.9	4337.6					2.3
1418.0	73	Padamate O	-7	1310.0	3930.0	85.1	-0.8	0.7	90.0	4.5	2.6	5.0	7.9
1509.0	74		-7	1401.0	4203.0	91.0	5.1	25.7					8.4
1554.0	75		-7	1446.0	4338.0	93.9	8.0	63.9					8.7
104.0	76	Padamate O	-8	-4.0	-12.0	-0.3	-86.2	7429.0	44.8	41.0	23.7	91.6	0.6
949.0	77		-8	841.0	2523.0	54.6	-31.3	979.9					5.3
1339.0	78		-8	1231.0	3693.0	80.0	-6.0	35.6					7.5
1582.0	79	Padamate O	-9	1474.0	4422.0	95.7	9.8	96.3	102.1	5.9	3.4	5.8	8.8
1696.0	80		-9	1588.0	4764.0	103.2	17.2	296.5					9.5
1761.0	81		-9	1653.0	4959.0	107.4	21.4	459.7					9.8
1693.0	82	Padamate O	-10	1585.0	4755.0	103.0	17.0	289.8	104.1	1.0	0.6	1.0	9.5
1716.0	83		-10	1608.0	4824.0	104.4	18.5	342.9					9.6
1724.0	84		-10	1616.0	4848.0	105.0	19.0	362.4					9.6

APPENDIX 1 Raw and Normalized Data Valid Run 3 – February 16, 2013

Experiment Date:	16-Feb-13	Study Number:	9070-100794ARB	Assays Conducted by:	[redacted]
Test substance:	Ensulizole				
3/6/2013 15:22					

ug protein/assay tube = 660.0

Tube	Sample Type	DPM (1mL)	Specific Binding DPM (100 uL) - NSB	Total Specific Binding (300 uL)	Mean
1	Total Activity (Master Mix)	57685	--	57685.0	56998.0
2		56378	--	56378.0	
3		56098	--	56098.0	
4		57264	--	57264.0	
5		56124	--	56124.0	
6		58439	--	58439.0	
7	Total Binding (Solvent Control)	1990	1871.0	5613	5510.5
8		1973	1854.0	5562	
9		1972	1853.0	5559	
10		2016	1897.0	5691	
11		1839	1720.0	5160	
12		1945	1826.0	5476	

DPM (1mL) from LSC	Tube	Sample Type	Concentration log[M]	Specific Binding DPM (1mL) - NSB	Total Specific Binding (3mL)	Specific Binding (%)	Residual	Squared Residual	Mean Specific Binding (%)	Standard Deviation	SEM	% CV	% Ligand Bound vs. Total Activity
99.0	13	R1881 (NSB)	-6	-20.0	-60.0	-1.1	-1.2	1.4	0.0	1.3	0.6	-1.8E+18	0.5
103.0	14		-6	-16.0	-48.0	-0.9	-1.0	0.9					0.5
96.0	15		-6	-23.0	-69.0	-1.3	-1.4	1.8					0.5
138.0	16		-6	19.0	57.0	1.0	0.9	0.9					0.7
120.0	17		-6	1.0	3.0	0.1	0.0	0.0					0.6
158.0	18		-6	39.0	117.0	2.1	2.0	4.1					0.8
122.0	19	R1881	-7	3.0	9.0	0.2	0.0	0.0	0.0	0.2	0.1	-526.8	0.6
118.0	20		-7	-1.0	-3.0	-0.1	-0.2	0.1					0.6
115.0	21		-7	-4.0	-12.0	-0.2	-0.4	0.2					0.6
151.0	22	R1881	-8	32.0	96.0	1.7	0.7	0.4	1.6	0.7	0.4	46.0	0.8
161.0	23		-8	42.0	126.0	2.3	1.2	1.4					0.8
134.0	24		-8	15.0	45.0	0.8	-0.3	0.1					0.7
298.0	25	R1881	-9	187.0	561.0	9.1	-0.5	0.2	9.5	0.4	0.2	4.3	1.5
301.0	26		-9	182.0	546.0	9.9	0.3	0.1					1.6
292.0	27		-9	173.0	519.0	9.4	-0.2	0.0					1.5
1117.0	28	R1881	-10	998.0	2994.0	54.3	2.0	3.9	52.4	3.6	2.1	6.8	5.9
1005.0	29		-10	886.0	2658.0	48.2	-4.1	16.9					5.3
1121.0	30		-10	1002.0	3006.0	54.6	2.2	4.8					5.9
1772.0	31	R1881	-11	1653.0	4959.0	90.0	-1.9	3.7	91.9	1.7	1.0	1.8	9.3
1822.0	32		-11	1703.0	5109.0	92.7	0.8	0.7					9.6
1827.0	33		-11	1708.0	5124.0	93.0	1.1	1.2					9.6
177.0	37	Dexamethasone	-3	58.0	174.0	3.2	-1.1	1.2	4.4	2.2	1.3	49.1	0.9
178.0	38		-3	59.0	177.0	3.2	-1.1	1.1					0.9
247.0	39		-3	128.0	384.0	7.0	2.7	7.3					1.3
436.0	40	Dexamethasone	-4	317.0	951.0	17.3	-3.7	13.4	20.6	3.0	1.7	14.4	2.3
539.0	41		-4	420.0	1260.0	22.9	2.0	3.8					2.8
518.0	42		-4	399.0	1197.0	21.7	0.8	0.7					2.7
1441.0	43	Dexamethasone	-5	1322.0	3966.0	72.0	-1.0	0.9	73.4	1.8	1.0	2.4	7.6
1503.0	44		-5	1384.0	4152.0	75.3	2.4	5.8					7.9
1455.0	45		-5	1336.0	4008.0	72.7	-0.2	0.0					7.7
1896.0	46	Dexamethasone	-6	1777.0	5331.0	96.7	-0.6	0.4	96.2	1.5	0.9	1.6	10.0
1854.0	47		-6	1736.0	5205.0	94.5	-2.9	8.6					9.8
1907.0	48		-6	1786.0	5364.0	97.3	0.0	0.0					10.0
1960.0	49	Dexamethasone	-7	1841.0	5523.0	100.2	-0.3	0.1	100.6	2.2	1.3	2.2	10.3
2011.0	50		-7	1892.0	5676.0	103.0	2.5	6.1					10.6
1932.0	51		-7	1813.0	5439.0	98.7	-1.8	3.3					10.2
2025.0	52	Dexamethasone	-8	1906.0	5718.0	103.8	2.9	8.6	102.5	1.8	1.1	1.8	10.7
2018.0	53		-8	1899.0	5697.0	103.4	2.6	6.5					10.6
1963.0	54		-8	1844.0	5532.0	100.4	-0.4	0.2					10.3
1929.0	55	Dexamethasone	-9	1810.0	5430.0	98.5	-2.3	5.4	101.7	2.9	1.7	2.8	10.2
1997.0	56		-9	1878.0	5634.0	102.2	1.4	1.9					10.5
2033.0	57		-9	1914.0	5742.0	104.2	3.3	11.2					10.7
1898.0	58	Dexamethasone	-10	1779.0	5337.0	96.9	-4.0	16.1	99.2	2.5	1.4	2.5	10.0
1989.0	59		-10	1870.0	5610.0	101.8	0.9	0.9					10.5
1936.0	60		-10	1817.0	5451.0	98.9	-1.9	3.6					10.2
1818.0	61	Ensulizole	-3	1700.0	5100.0	92.6	-2.3	5.3	93.6	5.6	3.2	6.0	9.6
1747.0	62		-3	1628.0	4884.0	88.6	-6.2	38.7					9.2
1950.0	63		-3	1831.0	5493.0	99.7	4.8	23.3					10.3
1905.0	64	Ensulizole	-4	1786.0	5358.0	97.2	2.4	5.7	92.9	6.3	3.6	6.7	10.0
1879.0	65		-4	1760.0	5280.0	95.8	1.0	0.9					9.9
1694.0	66		-4	1575.0	4725.0	85.7	-9.1	82.9					8.9
2026.0	67	Ensulizole	-5	1907.0	5721.0	103.8	9.0	80.4	102.6	1.3	0.7	1.3	10.7
2008.0	68		-5	1889.0	5667.0	102.8	8.0	63.8					10.6
1979.0	69		-5	1860.0	5580.0	101.3	6.4	41.1					10.4
1985.0	70	Ensulizole	-6	1866.0	5598.0	101.6	6.7	45.4	101.5	1.5	0.9	1.5	10.4
2010.0	71		-6	1891.0	5673.0	102.9	8.1	65.5					10.6
1954.0	72		-6	1835.0	5505.0	99.9	5.0	25.5					10.3
1967.0	73	Ensulizole	-7	1868.0	5604.0	101.7	6.8	46.8	75.5	43.7	25.2	57.9	10.5
1951.0	74		-7	1832.0	5496.0	99.7	4.9	23.9					10.3
579.0	75		-7	460.0	1380.0	25.0	-69.8	4873.4					3.0
1930.0	76	Ensulizole	-8	1811.0	5433.0	98.6	3.7	14.0	95.3	5.8	3.4	6.1	10.2
1934.0	77		-8	1816.0	5445.0	98.8	4.0	15.7					10.2
1746.0	78		-8	1627.0	4881.0	88.6	-6.3	39.4					9.2
1972.0	79	Ensulizole	-9	1853.0	5559.0	100.9	6.0	36.3	100.5	0.6	0.4	0.6	10.4
1971.0	80		-9	1852.0	5556.0	100.8	6.0	35.7					10.4
1951.0	81		-9	1832.0	5496.0	99.7	4.9	23.9					10.3
1881.0	82	Ensulizole	-10	1762.0	5286.0	95.9	1.1	1.2	96.9	2.4	1.4	2.5	9.9
1948.0	83		-10	1829.0	5487.0	99.6	4.7	22.3					10.3
1865.0	84		-10	1746.0	5238.0	95.1	0.2	0.0					9.8

APPENDIX 1 Raw and Normalized Data Valid Run 3 – February 16, 2013 (continued)

1960.0	61	Avobenzone	-3	1841.0	5523.0	100.2	2.2	4.8	97.0	2.8	1.6	2.9	10.3
1867.0	62		-3	1748.0	5244.0	95.2	-2.9	8.3					9.8
1874.0	63		-3	1755.0	5265.0	95.5	-2.5	6.2					9.9
1788.0	64	Avobenzone	-4	1669.0	5007.0	90.9	-7.2	51.5	94.9	3.5	2.0	3.7	9.4
1904.0	65		-4	1785.0	5355.0	97.2	-0.9	0.7					10.0
1892.0	66		-4	1773.0	5319.0	96.5	-1.5	2.3					10.0
2015.0	67	Avobenzone	-5	1896.0	5688.0	103.2	5.2	26.8	103.0	3.1	1.8	3.0	10.6
1952.0	68		-5	1833.0	5499.0	99.8	1.8	3.1					10.3
2064.0	69		-5	1945.0	5835.0	105.9	7.8	61.6					10.9
1892.0	70	Avobenzone	-6	1773.0	5319.0	96.5	-1.5	2.3	98.7	1.9	1.1	1.9	10.0
1959.0	71		-6	1840.0	5520.0	100.2	2.1	4.5					10.3
1944.0	72		-6	1825.0	5475.0	99.4	1.3	1.7					10.2
1904.0	73	Avobenzone	-7	1785.0	5355.0	97.2	-0.9	0.7	95.8	1.2	0.7	1.3	10.0
1870.0	74		-7	1751.0	5253.0	95.3	-2.7	7.4					9.8
1861.0	75		-7	1742.0	5226.0	94.8	-3.2	10.3					9.8
1966.0	76	Avobenzone	-8	1847.0	5541.0	100.6	2.5	6.3	100.9	0.8	0.5	0.8	10.3
1961.0	77		-8	1842.0	5526.0	100.3	2.2	5.0					10.3
1988.0	78		-8	1869.0	5607.0	101.8	3.7	13.8					10.5
2029.0	79	Avobenzone	-9	1910.0	5730.0	104.0	5.9	35.3	104.1	0.8	0.5	0.8	10.7
2047.0	80		-9	1928.0	5784.0	105.0	6.9	47.9					10.8
2016.0	81		-9	1897.0	5691.0	103.3	5.2	27.4					10.6
1883.0	82	Avobenzone	-10	1764.0	5292.0	96.0	-2.0	4.0	90.1	10.2	5.9	11.4	9.9
1883.0	83		-10	1764.0	5292.0	96.0	-2.0	4.0					9.9
1557.0	84		-10	1438.0	4314.0	78.3	-19.8	390.2					8.2
726.0	61	Homosalate	-3	607.0	1821.0	33.0	5.2	27.2	28.2	6.5	3.8	23.1	3.8
501.0	62		-3	382.0	1146.0	20.8	-7.0	49.5					2.6
683.0	63		-3	564.0	1692.0	30.7	2.9	8.3					3.6
972.0	64	Homosalate	-4	853.0	2559.0	46.4	-12.0	143.2	57.0	9.1	5.3	16.0	5.1
1264.0	65		-4	1145.0	3435.0	62.3	3.9	15.5					6.7
1260.0	66		-4	1141.0	3423.0	62.1	3.7	13.8					6.6
1878.0	67	Homosalate	-5	1759.0	5277.0	95.8	8.1	65.9	92.9	3.8	2.2	4.0	9.9
1853.0	68		-5	1734.0	5202.0	94.4	6.8	45.7					9.8
1748.0	69		-5	1629.0	4887.0	88.7	1.0	1.1					9.2
1218.0	70	Homosalate	-6	1099.0	3297.0	59.8	-36.6	1341.6	83.6	20.6	11.9	24.6	6.4
1893.0	71		-6	1774.0	5322.0	96.6	0.1	0.0					10.0
1852.0	72		-6	1733.0	5199.0	94.3	-2.1	4.5					9.7
1896.0	73	Homosalate	-7	1777.0	5331.0	96.7	-1.3	1.8	99.6	3.4	2.0	3.4	10.0
1933.0	74		-7	1814.0	5442.0	98.8	0.7	0.5					10.2
2019.0	75		-7	1900.0	5700.0	103.4	5.4	28.9					10.6
1942.0	76	Homosalate	-8	1823.0	5469.0	99.2	0.9	0.8	99.4	1.1	0.6	1.1	10.2
1966.0	77		-8	1847.0	5541.0	100.6	2.2	5.0					10.3
1927.0	78		-8	1808.0	5424.0	98.4	0.1	0.0					10.1
2050.0	79	Homosalate	-9	1931.0	5793.0	105.1	6.8	45.7	103.0	2.1	1.2	2.0	10.8
1974.0	80		-9	1855.0	5565.0	101.0	2.6	6.9					10.4
2010.0	81		-9	1891.0	5673.0	102.9	4.6	21.0					10.6
1971.0	82	Homosalate	-10	1852.0	5556.0	100.8	2.4	6.0	99.7	1.2	0.7	1.2	10.4
1955.0	83		-10	1836.0	5508.0	100.0	1.6	2.5					10.3
1926.0	84		-10	1807.0	5421.0	98.4	0.0	0.0					10.1
1390.0	61	Padamate O	-3	1271.0	3813.0	69.2	-25.5	649.2	81.7	10.9	6.3	13.3	7.3
1720.0	62		-3	1601.0	4803.0	87.2	-7.5	56.4					9.1
1750.0	63		-3	1631.0	4893.0	88.8	-5.9	34.6					9.2
1795.0	64	Padamate O	-4	1676.0	5028.0	91.2	-3.4	11.8	92.7	1.5	0.9	1.6	9.4
1821.0	65		-4	1702.0	5106.0	92.7	-2.0	4.1					9.6
1850.0	66		-4	1731.0	5193.0	94.2	-0.4	0.2					9.7
1889.0	67	Padamate O	-5	1770.0	5310.0	96.4	1.7	2.8	99.1	3.2	1.8	3.2	9.9
2003.0	68		-5	1884.0	5652.0	102.6	7.9	62.3					10.5
1924.0	69		-5	1805.0	5415.0	98.3	3.6	12.9					10.1
1971.0	70	Padamate O	-6	1852.0	5556.0	100.8	6.2	37.8	103.4	2.3	1.3	2.2	10.4
2034.0	71		-6	1915.0	5745.0	104.3	9.6	91.8					10.7
2050.0	72		-6	1931.0	5793.0	105.1	10.5	109.3					10.8
1950.0	73	Padamate O	-7	1831.0	5493.0	99.7	5.0	25.1	100.0	0.7	0.4	0.7	10.3
1946.0	74		-7	1827.0	5481.0	99.5	4.8	23.0					10.2
1971.0	75		-7	1852.0	5556.0	100.8	6.2	37.8					10.4
1552.0	76	Padamate O	-8	1433.0	4299.0	78.0	-16.7	277.5	91.2	11.5	6.7	12.6	8.2
1946.0	77		-8	1827.0	5481.0	99.5	4.8	23.0					10.2
1884.0	78		-8	1765.0	5295.0	96.1	1.4	2.0					9.9
1907.0	79	Padamate O	-9	1788.0	5364.0	97.3	2.7	7.1	97.0	0.6	0.4	0.7	10.0
1887.0	80		-9	1768.0	5304.0	96.3	1.6	2.5					9.9
1908.0	81		-9	1789.0	5367.0	97.4	2.7	7.4					10.0
1912.0	82	Padamate O	-10	1793.0	5379.0	97.6	2.9	8.6	92.3	9.2	5.3	9.9	10.1
1620.0	83		-10	1501.0	4503.0	81.7	-13.0	167.9					8.5
1912.0	84		-10	1793.0	5379.0	97.6	2.9	8.6					10.1

APPENDIX 2 Rat Prostate Cytosol Preparation and Information

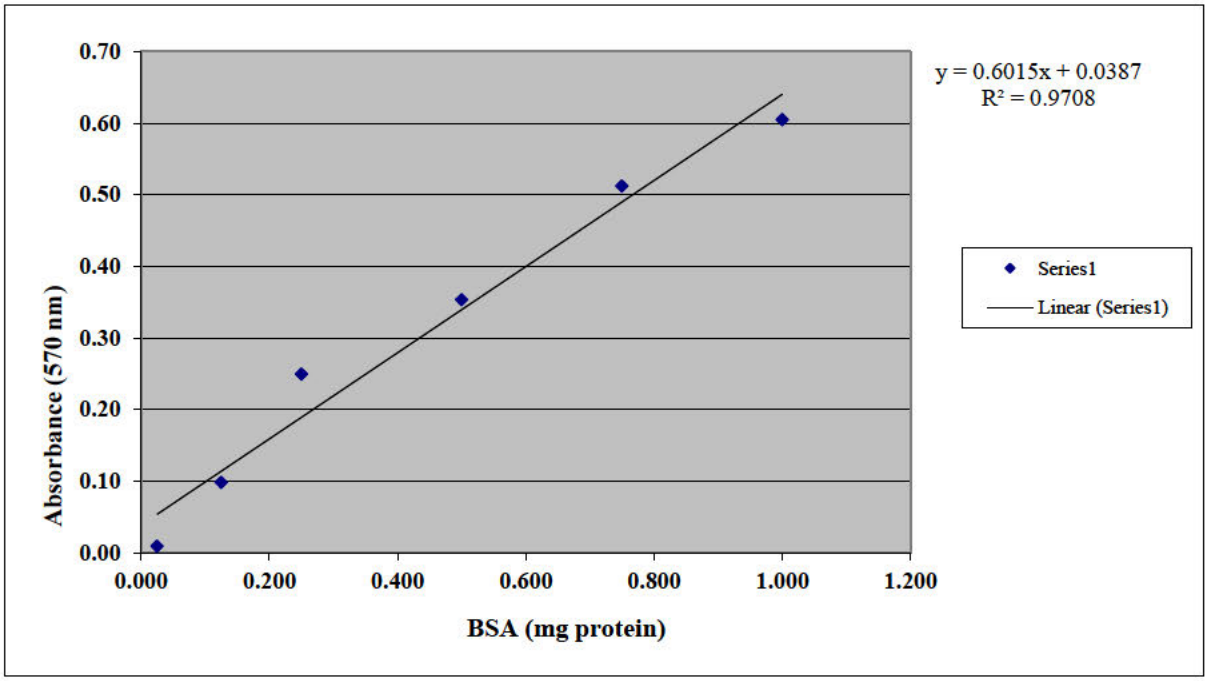
Runs 1 and 2 used cytosol isolated on February 01, 2013 and qualified on February 2-5, 2013. Run 3 used cytosol isolated on April 26, 2012 and qualified February 6-8, 2013.

February 01, 2013 cytosol preparation.

Supplier	Charles River Laboratories
Strain	Sprague-Dawley
Age	80-90 days
Days after castration	1 (24 hours)
Protein Concentration	1.5 mg/mL
Method of Determination	Bradford Method
Supplier and Product	Thermo Scientific Coomassie Bradford Protein Kit
Catalog Number	23200
Batch/Lot Number	NK175919
Method of Transport	FedEx – priority overnight
Conditions of Transport	Dry Ice

Rat prostate glands were purchased from Charles River Laboratories. Collection of rat prostrates was not performed according to GLP, though a QA inspection was performed on 01Feb13 and reported to management on 08Feb13. The cytosol preparation was performed on February 01, 2013. The homogenizer probe was pre-chilled by placing it in an ice cold beaker of low-salt TEDG buffer on ice. The prostate tissue was checked for healthy appearance (no fibrous, inflamed, edematous or infected appearance) and any tissues that appeared compromised were discarded and excess fascia was trimmed, if necessary. The prostate tissues were added to a beaker of low-salt TEDG buffer in ice bath, at 10 ml of buffer/g tissue. Prostates were minced with fine scissors until all pieces were small 1-2 mm cubes. Then the minced tissue was homogenized at ~4°C using a pre-chilled Polytron homogenizer. For the Polytron PT 10-35GT, setting 3, with 3 short 4 sec bursts of power spaced at 20 sec intervals was used. The homogenates were transferred to pre-cooled centrifuge tubes, balanced, and centrifuged at 30,000 x g for 30 minutes in a centrifuge cooled to ~4°C. The resulting supernatant contained the low-salt cytosolic receptors. The supernatant from all samples was pooled, gently mixed and aliquoted into labeled tubes and stored at approximately -80ºC. The cytosol preparations used in this study were thawed immediately prior to use in the assay and any leftover cytosol was discarded. The protein content for each batch of cytosol was determined using the Bradford method.

Calibration Curve



Raw Data Plate Map

	1	2	3	4	5	6	7	8	9	10	11	12
A						2	2	2		Neat cyto	Neat cyto	Neat cyto
B						1.5	1.5	1.5		2x cyto	2x cyto	2x cyto
C						1	1	1		3x cyto	3x cyto	3x cyto
D						0.75	0.75	0.75		5x cyto	5x cyto	5x cyto
E						0.5	0.5	0.5		10x cyto	10x cyto	10x cyto
F						0.25	0.25	0.25		water blank	water blank	water blank
G						0.125	0.125	0.125		water blank	water blank	water blank
H						0.025	0.025	0.025		water blank	water blank	water blank
						BSA standards (mg/mL)	BSA standards (mg/mL)	BSA standards (mg/mL)		cytosol samples	cytosol samples	cytosol samples

Raw Data

Plate Seq#: 9783
Comment:
Absorbance-A

Acquired: Friday, February 01, 2013 3:13 PM Temperature Min/Max: 0.0/0.0⁻C

File Report: C:\Fusion data files\MTT_(null)_02-01-13_2030.TXT

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.068	0.087	0.041	0.041	0.042	1.214	1.227	1.243	0.042	1.366	1.375	1.378
B	0.077	0.041	0.041	0.041	0.041	1.117	1.128	1.145	0.041	1.214	1.222	1.222
C	0.355	0.041	0.041	0.042	0.041	0.998	1.023	1.025	0.043	1.09	1.281	1.084
D	0.061	0.042	0.041	0.043	0.043	0.918	0.926	0.924	0.045	0.889	0.895	0.914
E	0.065	0.042	0.044	0.043	0.042	0.753	0.773	0.766	0.04	0.683	0.689	0.655
F	0.277	0.041	0.042	0.042	0.043	0.583	0.719	0.601	0.04	0.407	0.407	0.406
G	0.062	0.042	0.041	0.041	0.042	0.500	0.511	0.506	0.04	0.406	0.408	0.412
H	0.062	0.065	0.042	0.041	0.042	0.418	0.416	0.425	0.04	0.411	0.407	0.414

Protein Optimization

The optimal protein concentration for use in the assays was determined by incubating increasing concentrations of cytosol with 0.25 nM [^{3}H]-R1881. This allowed for the determination of the protein concentration that binds no more than 25-35% of the total radiolabel added.

Code (N=3 of each)	Receptor Dilution	Receptor concentration	Inert R1881	^{3}H-R1881	Cytosol
TB 0.25nM	undiluted	Conc 1	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:1.25	Conc 2	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:1.5	Conc 3	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:2	Conc 4	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:3	Conc 5	none	7.5 µL of 10 nM	300 µL
NSB 0.25nM	undiluted	Conc 1	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:1.25	Conc 2	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:1.5	Conc 3	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:2	Conc 4	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:3	Conc 5	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
TB 1.0 nM	undiluted	Conc 1	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:1.25	Conc 2	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:1.5	Conc 3	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:2	Conc 4	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:3	Conc 5	none	30 µL of 10 nM	300 µL
NSB 1.0 nM	undiluted	Conc 1	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:1.25	Conc 2	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:1.5	Conc 3	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:2	Conc 4	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:3	Conc 5	30 µL of 1 µM	30 µL of 10 nM	300 µL

Saturation Binding Methods

Once the proper protein concentration was determined, a saturation binding experiment measuring total and non-specific binding of [^{3}H]-R1881 was performed to demonstrate that the androgen receptor (AR) was present in reasonable concentrations and had the appropriate affinity for the native ligand. The conditions for the saturation binding experiment are summarized in Table 1.

TABLE A1. Summary of Conditions for Saturation Binding Experiment		
Source of receptor		Rat prostate cytosol
Concentration of radioligand (as serial dilutions)		0.25-10 nM
Concentration of non-labeled ligand (100X [radioligand])		25-1000 nM
Concentration of receptor		Sufficient to bind ~25-35% of radioligand at 0.25 nM
Temperature		~4ºC
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM
	Sodium Molybdate	1mM

The [^{3}H]-R1881 was manufactured on October 10, 2012 and the specific activity was 85.1 Ci/mmol. On the day of the assay the specific activity of the stock solution [^{3}H]-R1881 was adjusted for decay over time, and serial dilutions in low-salt TEDG + PMSF buffer were prepared to achieve the final concentrations of 0.25, 0.50, 0.70, 1.0, 1.5, 2.5, 5.0, and 10.0 nM. Solutions of non-labeled R1881 were prepared in a similar manner to achieve concentrations that were 100-fold greater than each respective radiolabeled concentration to result in final concentrations of 25, 50, 70, 100, 150, 250, 500 and 1000 nM. For each batch of cytosol, the optimal protein concentration was determined by calculating specific binding to differing amounts of protein per tube, using 0.25 nM radiolabeled R1881, until a concentration was reached that bound ~25-35% of the total radioactivity added. The protein concentration was 0.45 mg per assay tube for the three saturation binding experiments. Each assay consisted of three non-concurrent binding assay runs (February 02, 2013, February 04, 2013 and February 05, 2013), and each run contained three concurrent replicates at each concentration, resulting in the 72 sample tubes depicted in Table 2. QA inspection of Run 1 (Day 2) and Run 2 (Day 1) was performed on 04Feb13 and reported to management on 08Feb13.

Table A2. Saturation Binding Experiment Set-up Per Run

N=	Tube Type	Hot Initial Conc. (nM)	Hot R1881 Added (µL)	Hot Final Conc. (nM)[d]	Cold Initial Conc (µM)	Cold Added (µL)	Cold Final Conc. (nM)	Triamcinolone Added (µL)	Cytosol (µL)
3	TB[a]	10	7.5	0.25	--	--	--	50	300
3	TB	10	15	0.5	--	--	--	50	300
3	TB	10	21	0.7	--	--	--	50	300
3	TB	10	30	1	--	--	--	50	300
3	TB	10	45	1.5	--	--	--	50	300
3	TB	100	7.5	2.5	--	--	--	50	300
3	TB	100	15	5	--	--	--	50	300
3	TB	100	30	10	--	--	--	50	300
3	NSB[b]	10	7.5	0.25	1	7.5	25	50	300
3	NSB	10	15	0.5	1	15	50	50	300
3	NSB	10	21	0.7	1	21	70	50	300
3	NSB	10	30	1	1	30	100	50	300
3	NSB	10	45	1.5	1	45	150	50	300
3	NSB	100	7.5	2.5	10	7.5	250	50	300
3	NSB	100	15	5	10	15	500	50	300
3	NSB	100	30	10	10	30	1000	50	300
3	TA[c]	10	7.5	--	--	--	--	--	--
3	TA	10	15	--	--	--	--	--	--
3	TA	10	21	--	--	--	--	--	--
3	TA	10	30	--	--	--	--	--	--
3	TA	10	45	--	--	--	--	--	--
3	TA	100	7.5	--	--	--	--	--	--
3	TA	100	15	--	--	--	--	--	--
3	TA	100	30	--	--	--	--	--	--

a Total binding = [^{3}H]-R1881 bound to AR

b Non-specific binding = [^{3}H]-R1881 and 100-fold greater non-labeled bound to AR

c Total [^{3}H]-R1881 alone for dpm determination at each concentration

First, the necessary volumes of [^{3}H]-R1881, cold R1881 and 60 µM triamcinolone were added to every total binding tube and non-specific binding tube. These tubes were placed in a speed-vac to dry. While the tubes were drying, aliquots of each concentration of [^{3}H]-R1881 were added, in triplicate, to 14 mL scintillation cocktail in a 20 mL scintillation vial to determine total radioligand added. Once cytosol was added, the tubes were incubated at approximately 4°C, with gentle vortexing, for 20 hr 35 min, 19 hr 35 min and 19 hr 40 min for the first, second and third saturation binding experiments, respectively. To separate bound from free R1881, 500 µL of hydroxyapatite (HAP) slurry was added to fresh tubes. A 100 µL aliquot of each total binding and non-specific binding tube was added to the HAP tubes and they were vortexed (5 times with 4-minute intervals). Subsequently, the contents of each tube were washed three times as follows: 2 mL of ice cold TEDG +PMSF buffer was added, vortexed and centrifuged for 3 min at 700 x *g*. The supernatant decanted and discarded. The HAP pellet remaining in each tube was resuspended in 2 mL absolute ethanol to extract the [^{3}H]-R1881, followed by vortexing, and centrifugation for 10 min at 700 x *g*. 20 mL scintillation vials were filled with 14 mL scintillation cocktail and the entire supernatant was assessed by scintillation counting. The temperature was maintained at

approximately 4ºC throughout the assay prior to extraction with ethanol. This was repeated two more times for a total of three saturation binding runs.

Data Analysis

For the Saturation Binding Experiment, total binding and non-specific binding data were modeled via non-linear regression using Graph Pad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA), incorporating automatic outlier elimination according to the method of Motulsky and Brown (2006) implemented by using the ROUT procedure in Prism v. 5 with a Q value of 1.0. Scatchard plots were also generated using Graph Pad Prism v. 5. Receptor binding data plots were corrected for ligand depletion using the method of Swillens (1995). Parameters reported from the Saturation Binding Experiment (K_d and B_{max}), means and standard deviations, were calculated for each run and the means and standard errors were calculated for the composite three runs using Microsoft Excel 2010 (Redmond, WA; version 14.0.6123.5001).

Saturation Binding Results

Non-specific binding was only 10.0% of total binding (mean value for all three saturation binding runs). This is within the suggested range of 8.1% – 10.0% and is within historical CeeTox values. The mean dissociation constant (K_d) for [^{3}H]-R1881 was 0.536 ± 0.038 nM. The mean estimated B_{max} was 0.012 ± 0.0002 nM (4.657 ± 0.071 fmol/100µg) for the single batch of prostate cytosol that was prepared. Though both these values are slightly lower than the suggested values in the guideline, they are comparable to CeeTox historical data. Confidence in these numbers is high according to the goodness of fit (r^2 = 0.947 – 0.992) and the small variation among runs.

TABLE A3. Saturation Binding Experiment of R1881 with Androgen Receptor from Rat Prostate Cytosol

Parameter	Run 1	Run 2	Run 3	Runs 1-3 [a]
R^2 (unweighted)	0.992	0.947	0.969	0.947 – 0.992
B_{max} (nM)	0.011	0.012	0.013	0.012 ± 0.0002
B_{max} (fmol/100 µg protein)	4.526	4.679	4.768	4.657 ± 0.071
K_d (nM)	0.474	0.530	0.606	0.536 ± 0.038

a The range of R^2 is reported and the mean ± SEM is reported for the other parameters.
R^2 = Goodness of fit for curve calculated for specific binding

Figure A1. Binding of [^{3}H]-R1881 to the Androgen Receptor during the Saturation Binding Experiment, Run 1 (02-February-2013).

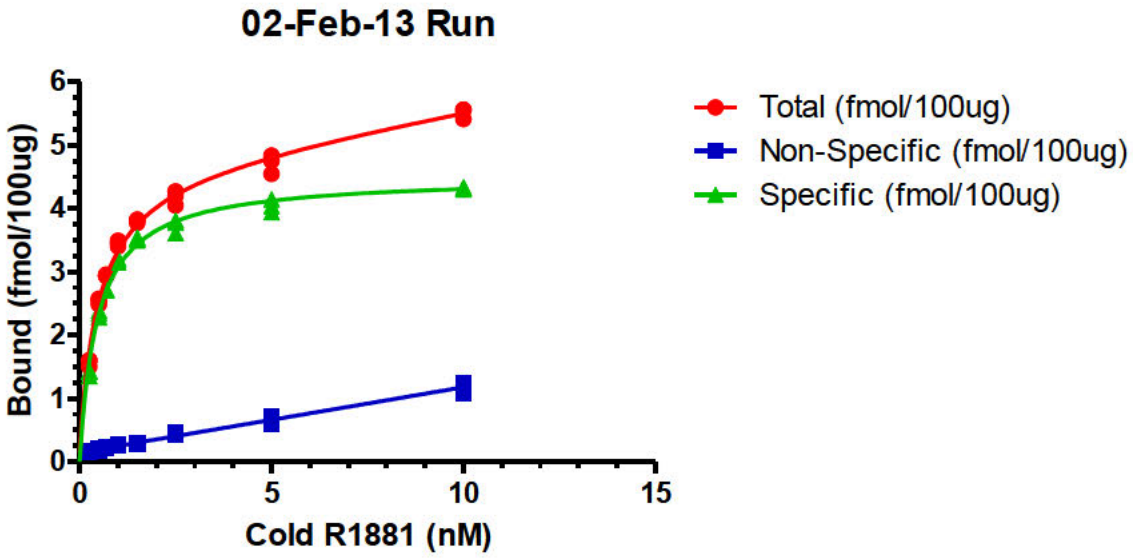


Figure A2. Scatchard Plot of the Binding of [^{3}H]-R1881 to the Androgen Receptor, Run 1 (02-February-2013).

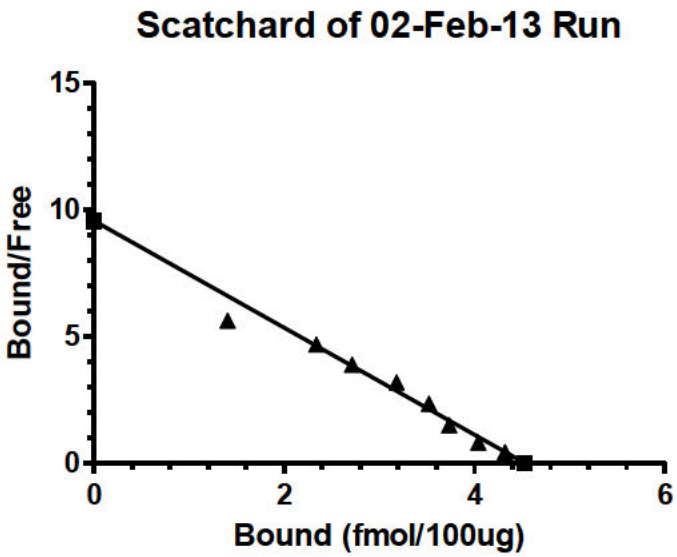


Figure A3. Binding of [^{3}H]-R1881 to the Androgen Receptor during the Saturation Binding Experiment, Run 2 (04-February-2013).

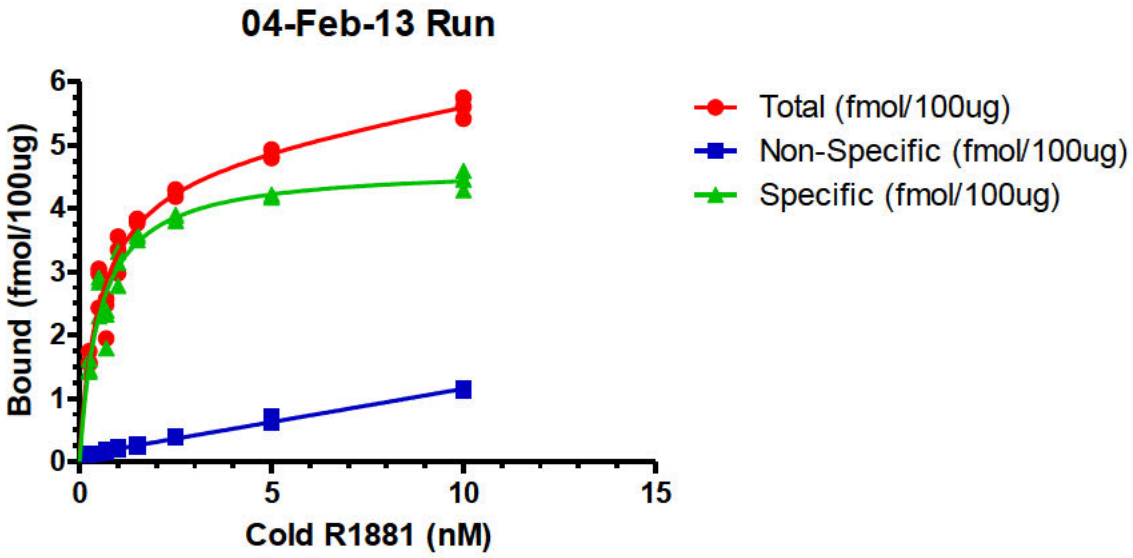


Figure A4. Scatchard Plot of the Binding of [^{3}H]-R1881 to the Androgen Receptor, Run 2 (04-February-2013).

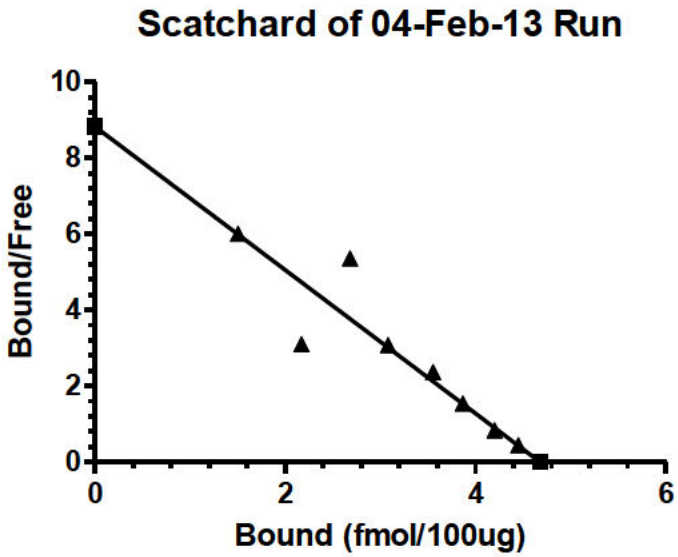


Figure A5. **Binding of [^{3}H]-R1881 to the Androgen Receptor during the Saturation Binding Experiment, Run 3 (05-February-2013).**

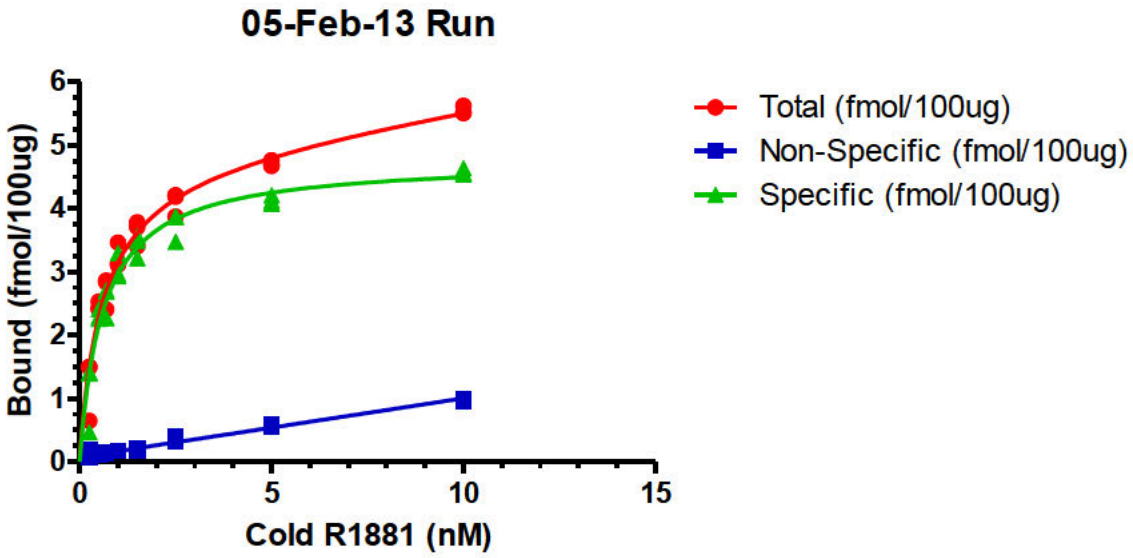
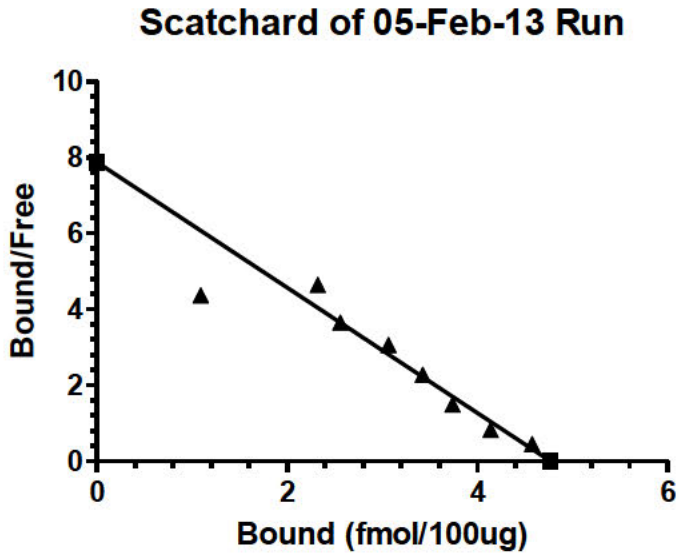


Figure A6. **Scatchard Plot of the Binding of [^{3}H]-R1881 to the Androgen Receptor, Run 3 (05-February-2013).**

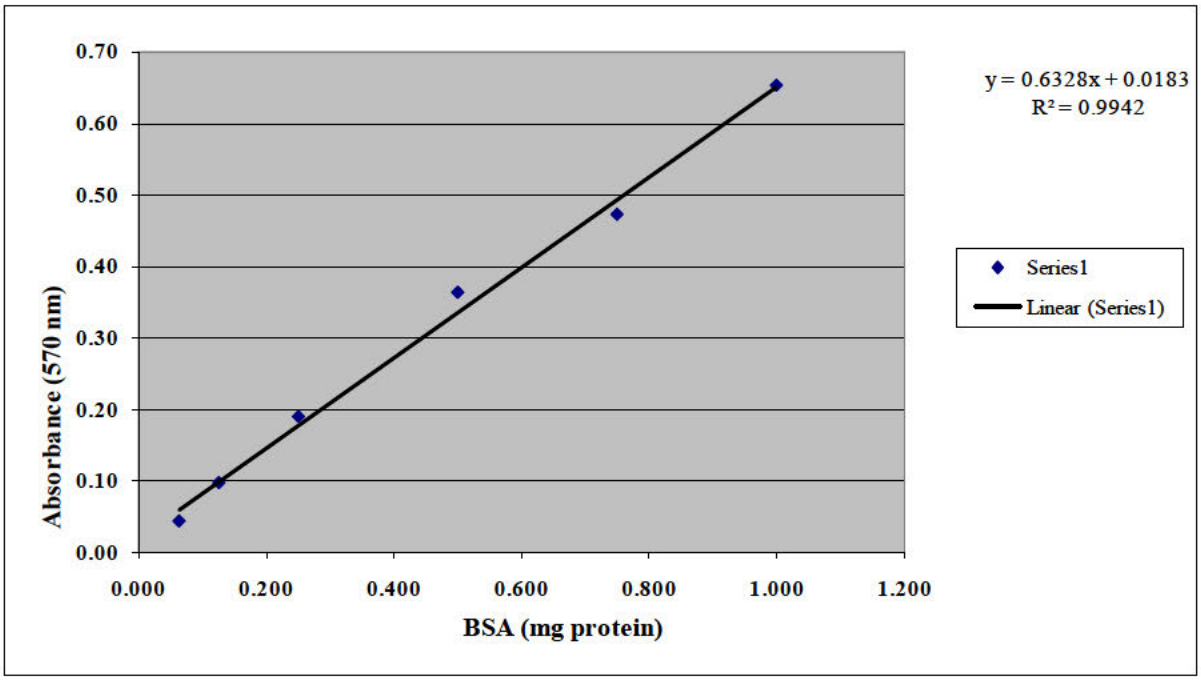


April 26, 2012 cytosol preparation.

Supplier	Charles River Laboratories
Strain	Sprague-Dawley
Age	80-90 days
Days after castration	1 (24 hours)
Protein Concentration	2.2 mg/mL
Method of Determination	Bradford Method
Supplier and Product	Bio-Rad Dye Reagent
Catalog Number	500-0205
Batch/Lot Number	200005735
Method of Transport	FedEx – priority overnight
Conditions of Transport	Dry Ice

Rat prostate glands were purchased from Charles River Laboratories. Collection of rat prostrates was not performed according to GLP. The cytosol preparation was performed on April 26, 2012. The homogenizer probe was pre-chilled by placing it in an ice cold beaker of low-salt TEDG buffer on ice. The prostate tissue was checked for healthy appearance (no fibrous, inflamed, edematous or infected appearance) and any tissues that appeared compromised were discarded and excess fascia was trimmed, if necessary. The prostate tissues were added to a beaker of low-salt TEDG buffer in ice bath, at 10 ml of buffer/g tissue. Prostates were minced with fine scissors until all pieces were small 1-2 mm cubes. Then the minced tissue was homogenized at ~4°C using a pre-chilled Polytron homogenizer. For the Polytron PT 10-35GT, setting 3, with 3 short 4 sec bursts of power spaced at 20 sec intervals was used. The homogenates were transferred to pre-cooled centrifuge tubes, balanced, and centrifuged at 30,000 x g for 30 minutes in a centrifuge cooled to ~4°C. The resulting supernatant contained the low-salt cytosolic receptors. The supernatant from all samples was pooled, gently mixed and aliquoted into labeled tubes and stored at approximately -80ºC. The cytosol preparations used in this study were thawed immediately prior to use in the assay and any leftover cytosol was discarded. The protein content for each batch of cytosol was determined using the Bradford method.

Calibration Curve



Raw Data Plate Map

The green color refers to the protein optimization and the blue color is the bovine serum albumin (BSA) standard curve used to determine protein concentration of the isolated prostate cytosol.

	1	2	3	4	5	6	7	8	9	10	11	12
A	neat cyto	neat cyto	neat cyto	water	empty	empty	empty	empty	empty	2	2	2
B	2X cyto	2X cyto	2X cyto	water	empty	empty	empty	empty	empty	1	1	1
C	3X cyto	3X cyto	3X cyto	water	empty	empty	empty	empty	empty	0.5	0.5	0.5
D	10X cyto	10X cyto	10X cyto	water	empty	empty	empty	empty	empty	0.25	0.25	0.25
E	20X cyto	20X cyto	20X cyto	water	empty	empty	empty	empty	empty	0.1	0.1	0.1
F	40X cyto	40X cyto	40X cyto	water	empty	empty	empty	empty	empty	0.05	0.05	0.05
G	buffer	buffer	buffer	water	empty	empty	empty	empty	empty	0.025	0.025	0.025
H	water	water	water	water	empty	empty	empty	empty	empty	0.01	0.01	0.01

Raw Data

Absorbance values at a wavelength of 570 nm.

	1	2	3	4	5	6	7	8	9	10	11	12
A	3.310	3.310	3.310	0.140	0.044	0.041	0.040	0.041	0.042	2.065	2.125	2.071
B	2.708	2.611	2.590	0.142	0.041	0.041	0.042	0.049	0.043	1.300	1.177	1.230
C	2.344	2.348	2.336	0.141	0.042	0.041	0.041	0.041	0.041	0.658	0.724	0.794
D	0.848	0.969	0.847	0.315	0.042	0.042	0.042	0.039	0.042	0.455	0.457	0.457
E	0.526	0.512	0.514	0.139	0.050	0.044	0.038	0.040	0.038	0.253	0.279	0.280
F	0.354	0.342	0.356	0.143	0.043	0.041	0.039	0.040	0.040	0.200	0.203	0.209
G	0.138	0.145	0.139	0.142	0.043	0.040	0.037	0.040	0.038	0.176	0.181	0.196
H	0.144	0.144	0.144	0.143	0.042	0.042	0.041	0.039	0.039	0.159	0.158	0.156

Protein Optimization

The optimal protein concentration for use in the assays was determined by incubating increasing concentrations of cytosol with 0.25 nM [^{3}H]-R1881. This allowed for the determination of the protein concentration that binds no more than 25-35% of the total radiolabel added.

Code (N=3 of each)	Receptor Dilution	Receptor concentration	Inert R1881	^{3}H-R1881	Cytosol
TB 0.25nM	undiluted	Conc 1	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:1.25	Conc 2	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:1.5	Conc 3	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:2	Conc 4	none	7.5 µL of 10 nM	300 µL
TB 0.25nM	1:3	Conc 5	none	7.5 µL of 10 nM	300 µL
NSB 0.25nM	undiluted	Conc 1	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:1.25	Conc 2	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:1.5	Conc 3	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:2	Conc 4	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
NSB 0.25nM	1:3	Conc 5	7.5 µL of 1 µM	7.5 µL of 10 nM	300 µL
TB 1.0 nM	undiluted	Conc 1	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:1.25	Conc 2	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:1.5	Conc 3	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:2	Conc 4	none	30 µL of 10 nM	300 µL
TB 1.0 nM	1:3	Conc 5	none	30 µL of 10 nM	300 µL
NSB 1.0 nM	undiluted	Conc 1	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:1.25	Conc 2	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:1.5	Conc 3	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:2	Conc 4	30 µL of 1 µM	30 µL of 10 nM	300 µL
NSB 1.0 nM	1:3	Conc 5	30 µL of 1 µM	30 µL of 10 nM	300 µL

Saturation Binding Methods

Once the proper protein concentration was determined, a saturation binding experiment measuring total and non-specific binding of [^{3}H]-R1881 was performed to demonstrate that the androgen receptor (AR) was present in reasonable concentrations and had the appropriate affinity for the native ligand. The conditions for the saturation binding experiment are summarized in Table 1.

TABLE A4. Summary of Conditions for Saturation Binding Experiment		
Source of receptor		Rat prostate cytosol
Concentration of radioligand (as serial dilutions)		0.25-10 nM
Concentration of non-labeled ligand (100X [radioligand])		25-1000 nM
Concentration of receptor		Sufficient to bind ~25-35% of radioligand at 0.25 nM
Temperature		~4ºC
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10%
	Phenylmethylsulfonyl fluoride	1 mM
	DTT	1 mM
	Sodium Molybdate	1mM

The [^{3}H]-R1881 was manufactured on October 10, 2012 and the specific activity was 85.1 Ci/mmol. On the day of the assay the specific activity of the stock solution [^{3}H]-R1881 was adjusted for decay over time, and serial dilutions in low-salt TEDG + PMSF buffer were prepared to achieve the final concentrations of 0.25, 0.50, 0.70, 1.0, 1.5, 2.5, 5.0, and 10.0 nM. Solutions of non-labeled R1881 were prepared in a similar manner to achieve concentrations that were 100-fold greater than each respective radiolabeled concentration to result in final concentrations of 25, 50, 70, 100, 150, 250, 500 and 1000 nM. For each batch of cytosol, the optimal protein concentration was determined by calculating specific binding to differing amounts of protein per tube, using 0.25 nM radiolabeled R1881, until a concentration was reached that bound ~25-35% of the total radioactivity added. The protein concentration was 0.660 mg per assay tube for the three saturation binding experiments. Each assay consisted of three non-concurrent binding assay runs (February 02, 2013, February 04, 2013 and February 05, 2013), and each run contained three concurrent replicates at each concentration, resulting in the 72 sample tubes depicted in Table 2.

Table A5. Saturation Binding Experiment Set-up Per Run									
N=	Tube Type	Hot Initial Conc. (nM)	Hot R1881 Added (µL)	Hot Final Conc. (nM)[d]	Cold Initial Conc (µM)	Cold Added (µL)	Cold Final Conc. (nM)	Triamcinolone Added (µL)	Cytosol (µL)
3	TB[a]	10	7.5	0.25	--	--	--	50	300
3	TB	10	15	0.5	--	--	--	50	300
3	TB	10	21	0.7	--	--	--	50	300
3	TB	10	30	1	--	--	--	50	300
3	TB	10	45	1.5	--	--	--	50	300
3	TB	100	7.5	2.5	--	--	--	50	300
3	TB	100	15	5	--	--	--	50	300
3	TB	100	30	10	--	--	--	50	300
3	NSB[b]	10	7.5	0.25	1	7.5	25	50	300
3	NSB	10	15	0.5	1	15	50	50	300
3	NSB	10	21	0.7	1	21	70	50	300
3	NSB	10	30	1	1	30	100	50	300
3	NSB	10	45	1.5	1	45	150	50	300
3	NSB	100	7.5	2.5	10	7.5	250	50	300
3	NSB	100	15	5	10	15	500	50	300
3	NSB	100	30	10	10	30	1000	50	300
3	TA[c]	10	7.5	--	--	--	--	--	--
3	TA	10	15	--	--	--	--	--	--
3	TA	10	21	--	--	--	--	--	--
3	TA	10	30	--	--	--	--	--	--
3	TA	10	45	--	--	--	--	--	--
3	TA	100	7.5	--	--	--	--	--	--
3	TA	100	15	--	--	--	--	--	--
3	TA	100	30	--	--	--	--	--	--

a Total binding = [^{3}H]-R1881 bound to AR

b Non-specific binding = [^{3}H]-R1881 and 100-fold greater non-labeled bound to AR

c Total [^{3}H]-R1881 alone for dpm determination at each concentration

First, the necessary volumes of [^{3}H]-R1881, cold R1881 and 60 µM triamcinolone were added to every total binding tube and non-specific binding tube. These tubes were placed in a speed-vac to dry. While the tubes were drying, aliquots of each concentration of [^{3}H]-R1881 were added, in triplicate, to 14 mL scintillation cocktail in a 20 mL scintillation vial to determine total radioligand added. Once cytosol was added, the tubes were incubated at approximately 4°C, with gentle vortexing, for 20 hr 0 min, 20 hr 30 min and 19 hr 30 min for the first, second and third saturation binding experiments, respectively. To separate bound from free R1881, 500 µL of hydroxyapatite (HAP) slurry was added to fresh tubes. A 100 µL aliquot of each total binding and non-specific binding tube was added to the HAP tubes and they were vortexed (5 times with 4-minute intervals). Subsequently, the contents of each tube were washed three times as follows: 2 mL of ice cold TEDG +PMSF buffer was added, vortexed and centrifuged for 3 min at 700 x *g*. The supernatant decanted and discarded. The HAP pellet remaining in each tube was resuspended in 2 mL absolute ethanol to extract the [^{3}H]-R1881, followed by vortexing, and centrifugation for 10 min at 700 x *g*. 20 mL scintillation vials were filled with 14 mL scintillation cocktail and the entire supernatant was assessed by scintillation counting. The temperature was maintained at

approximately 4ºC throughout the assay prior to extraction with ethanol. This was repeated two more times for a total of three saturation binding runs.

Data Analysis

For the Saturation Binding Experiment, total binding and non-specific binding data were modeled via non-linear regression using Graph Pad Prism v. 5 (GraphPad Software, Inc., La Jolla, CA), incorporating automatic outlier elimination according to the method of Motulsky and Brown (2006) implemented by using the ROUT procedure in Prism v. 5 with a Q value of 1.0. Scatchard plots were also generated using Graph Pad Prism v. 5. Receptor binding data plots were corrected for ligand depletion using the method of Swillens (1995). Parameters reported from the Saturation Binding Experiment (K_d and B_{max}), means and standard deviations, were calculated for each run and the means and standard errors were calculated for the composite three runs using Microsoft Excel 2010 (Redmond, WA; version 14.0.6123.5001).

Saturation Binding Results

Non-specific binding was only 10.0% of total binding (mean value for all three saturation binding runs). This is within the suggested range of 8.1% – 10.0% and is within historical CeeTox values. The mean dissociation constant (K_d) for [^{3}H]-R1881 was 0.432 ± 0.017 nM. The mean estimated B_{max} was 0.008 ± 0.0001 nM (2.583 ± 0.036 fmol/100µg) for the single batch of prostate cytosol that was prepared. Though the K_d values are slightly lower than the suggested values in the guideline, they are comparable to CeeTox historical data. Confidence in these numbers is high according to the goodness of fit (r^2 = 0.974 – 0.995) and the small variation among runs.

TABLE A6. Saturation Binding Experiment of R1881 with Androgen Receptor from Rat Prostate Cytosol

Parameter	Run 1	Run 2	Run 3	Runs 1-3 [a]
R^2 (unweighted)	0.992	0.947	0.969	0.947 – 0.992
B_{max} (nM)	0.011	0.012	0.013	0.012 ± 0.0002
B_{max} (fmol/100 µg protein)	4.526	4.679	4.768	4.657 ± 0.071
K_d (nM)	0.474	0.530	0.606	0.536 ± 0.038

a The range of R^2 is reported and the mean ± SEM is reported for the other parameters.
R^2 = Goodness of fit for curve calculated for specific binding

Figure A7. Binding of [^{3}H]-R1881 to the Androgen Receptor during the Saturation Binding Experiment, Run 1 (06-Feb-13).

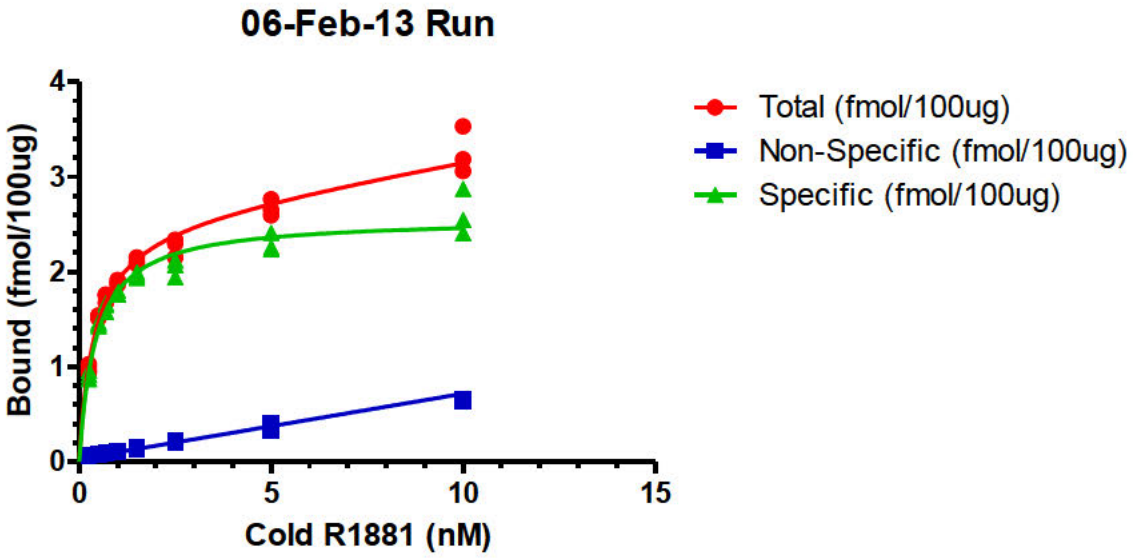


Figure A8. Scatchard Plot of the Binding of [^{3}H]-R1881 to the Androgen Receptor, Run 1 (06-Feb-13).

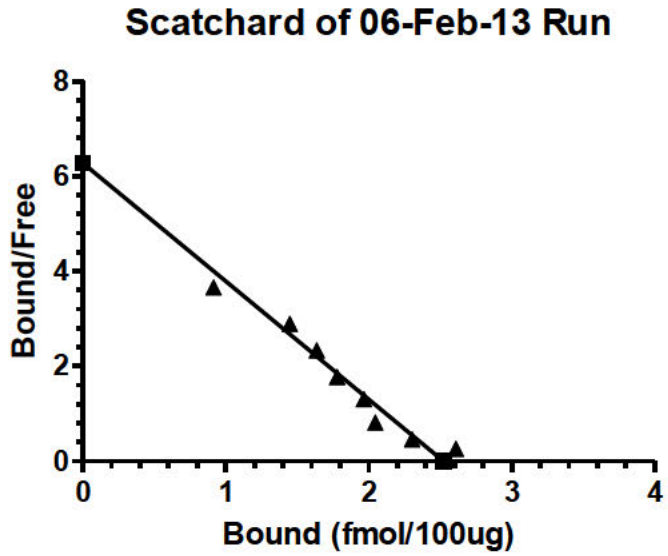


Figure A9. Binding of [^{3}H]-R1881 to the Androgen Receptor during the Saturation Binding Experiment, Run 2 (07-Feb-13).

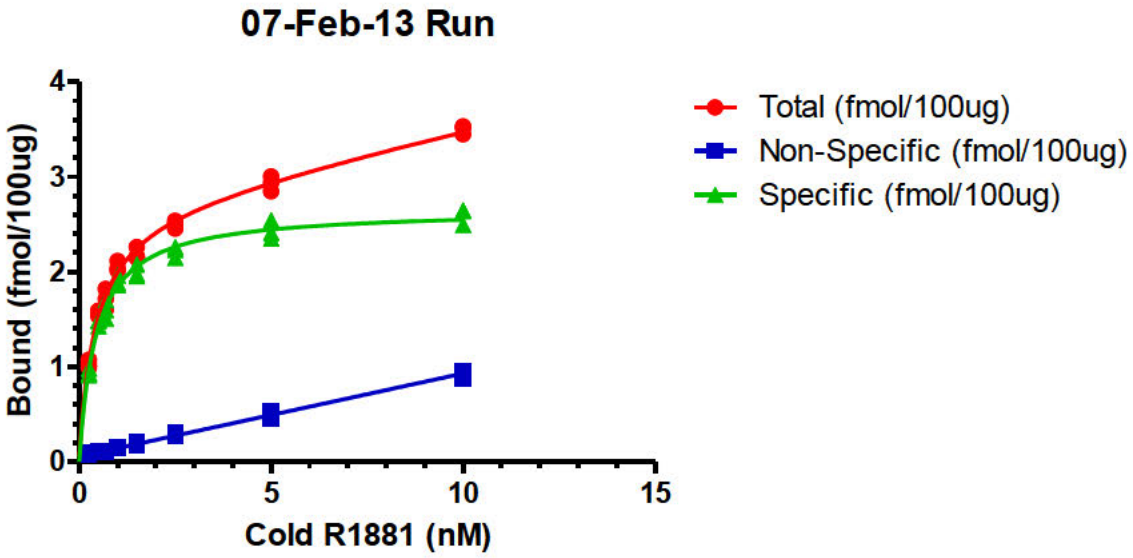


Figure A10. Scatchard Plot of the Binding of [^{3}H]-R1881 to the Androgen Receptor, Run 2 (07-Feb-13).

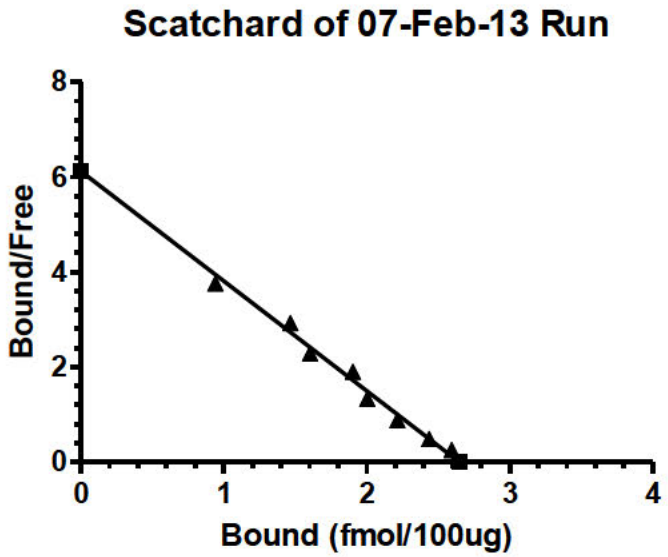


Figure A11. Binding of [^{3}H]-R1881 to the Androgen Receptor during the Saturation Binding Experiment, Run 3 (08-Feb-13).

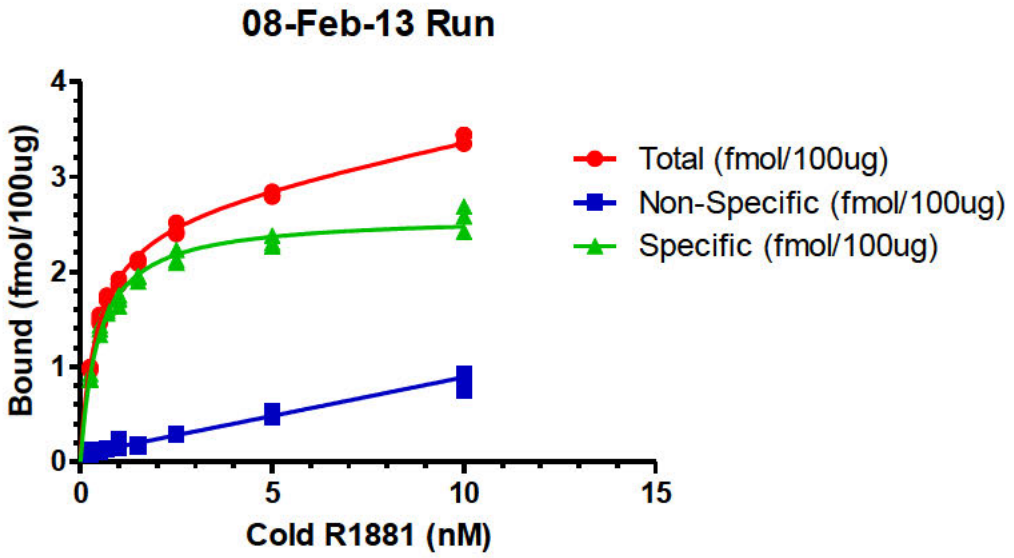
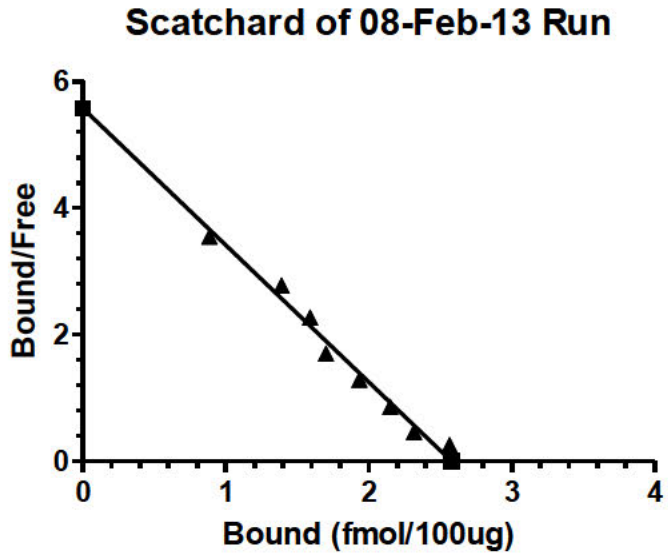


Figure A12. Scatchard Plot of the Binding of [^{3}H]-R1881 to the Androgen Receptor, Run 3 (08-Feb-13).



APPENDIX 3 Deviation Forms

CeeTox
In vitro models to predict toxicity

Form #: SOP-1003-F-1.2

Deviation and Investigation

Study Number (if applicable): 9070-100794ARB

SOP Number (if applicable): N/A

Equipment Serial Number (if applicable): N/A

Date of Reporting: 18-Feb-13 Reporting Associate: [redacted]

Date of Occurrence: 11-Feb-13, 14-Feb-13 and 16-Feb-13 Associate Involved: [redacted]

Description of Deviation:

Test materials diluted in 96-well plate instead of tubes. Smaller volumes prepared than protocol states.

Signature [redacted] Date: 18-Feb-13

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:

Diluted test materials into 96-well plate instead of tubes because lower volumes were prepared.

Action Taken and Determination of Impact on Study Data and/or Facility Compliance:

None.

Risk Associated with deviation:

None.

Signature [redacted] Date: 18 Feb 2013

SD/PI/Management Acknowledgement

Signature [redacted] Date: 18 Feb 2013

Quality Assurance Review

If study specific, sponsor notified on: 18 Feb 13 By: [redacted] 22 Feb 13

See Attached Documentation (email documentation is sufficient) ☒

Standard Operating Procedure Page 1 of 1

Form #: SOP-1003-F-1.2

CeeTox
In vitro models to predict toxicity

Deviation and Investigation

Study Number (if applicable): 9070-100794ARB

SOP Number (if applicable): N/A

Equipment Serial Number (if applicable): N/A

Date of Reporting: 18-Feb-13 Reporting Associate: [redacted]

Date of Occurrence: 11-Feb-13, 14-Feb-13 and 16-Feb-13 Associate Involved: [redacted]

Description of Deviation:

Centrifuged tubes for ~3 min instead of ~10 min, except for the last centrifugation (after Ethanol added)

Signature [redacted] Date: 18-Feb-13

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:

10 minutes too long, HAP more easily washed/resuspended after 3 min centrifugation

Action Taken and Determination of Impact on Study Data and/or Facility Compliance:

None.

Risk Associated with deviation:

None.

Signature [redacted] Date: 18 Feb 2013

SD/PI/Management Acknowledgement

Signature [redacted] Date: 18Feb2013

Quality Assurance Review

If study specific, sponsor notified on: 18 Feb 13 By: [redacted] 22 Feb 13

See Attached Documentation (email documentation is sufficient) ☒

Standard Operating Procedure Page 1 of 1

Form #: SOP-1003-F-1.2

CeeTox
In vitro models to predict toxicity

Deviation and Investigation

Study Number (if applicable): 9070-100794ARB

SOP Number (if applicable): N/A

Equipment Serial Number (if applicable): N/A

Date of Reporting: 14-Mar-13 Reporting Associate: [redacted]

Date of Occurrence: 11-Feb-13, 14-Feb-13 and 16-Feb-13 Associate Involved: [redacted]

Description of Deviation:

Triamcinolone acetonide was prepared in 100% ethanol per CeeTox SOP and OPPTS guideline (protocol states DMSO)

Signature [redacted] Date: 14-Mar-13

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:

Triamcinolone acetonide was prepared in 100% ethanol per CeeTox SOP and OPPTS guideline (protocol states DMSO)

Action Taken and Determination of Impact on Study Data and/or Facility Compliance:

None.

Risk Associated with deviation:

None.

Signature [redacted] Date: 14-Mar-13

SD/PI/Management Acknowledgement

Signature [redacted] Date: 14-Mar-13

Quality Assurance Review

If study specific, sponsor notified on: 14 March 2013 By: [redacted]

See Attached Documentation (email documentation is sufficient) ☒

Standard Operating Procedure Page 1 of 1

APPENDIX 4 Certificates of Analysis

9070-100794ARB

SIGMA-ALDRICH®

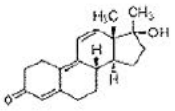
sigma-aldrich.com

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:
R1881 - ≥98% (HPLC)

Product Number:	**R0908**
Batch Number:	**112M4617V**
Brand:	SIGMA
CAS Number:	965-93-5
Formula:	C19H24O2
Formula Weight:	284.39 g/mol
Storage Temperature:	Store at 2 - 8 °C
Quality Release Date:	20 NOV 2012



Test	Specification	Result
Appearance (Color)	Light Yellow to Yellow	faint yellow
Appearance (Form)	Powder	Powder
Elemental Composition C19H24O2	Pass	Pass
Purity (HPLC)	≥ 98 %	99 %
Specific Rotation (C= 0.5,Ethanol)	-62.0 - -42.0 °	-52.9 °
Identity	Confirmed	Conforms

[redacted]
[redacted] QC Team Leader
Quality Control
Natick, Massachusetts US

Version Number: 1

9070-100794ARB

SIGMA-ALDRICH

SIGMA Life Science

Industriestrasse 25, CH-9471 Buchs (SG), Switzerland
Tel: +41 81 755 2511 Fax: +41 81 756 5449

Certificate of Analysis

Product Name:	DEXAMETHASONE >= 98 % HPLC, powder
Product Number:	D1756
Product Brand:	Sigma
Molecular Formula:	$C_{22}H_{29}FO_5$
Molecular Mass:	392.46
CAS Number:	50-02-2

TEST	SPECIFICATION	LOT 1419230 RESULTS
APPEARANCE (COLOR)	WHITE TO OFF-WHITE	WHITE
APPEARANCE (FORM)	POWDER	POWDER
PURITY (HPLC AREA %)	≥ 98 %	98.9 %
SOLUBILITY (COLOR)	COLORLESS	COLORLESS
SOLUBILITY (TURBIDITY)	CLEAR	CLEAR (<3.5 NTU)
SOLUBILITY (METHOD)	25MG/ML OF METHANOL	25MG/ML OF METHANOL
WAVELENGHT (1) (UV)	LAMBDA MAX 239 TO 241 NM	LAMBDA MAX 239.2 NM
MOLAR ABSORBANCY INDEX (1)	EMM = 15.0 TO 15.5	EMM = 15.3
SOLVENT (UV)	METHANOL	METHANOL

QC RELEASE DATE 12/DEC/08
RECOMMENDED RETEST DATE NOV/13

[redacted] Manager
Quality Control
Buchs, Switzerland

TECHNICAL DATA SHEET ^{3}H 9070-100794ARB

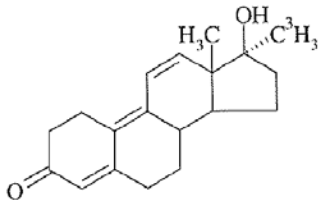
Caution: For Laboratory Use. A product for research purposes only.

METHYLTRIENOLONE, [17α-METHYL-^{3}H]- (METRIBOLONE, R1881*)

Product Number: NET590

LOT SPECIFIC INFORMATION

Lot Number:	1001606
Specific Activity:	85.1 Ci/mmol
	3149 GBq/mmol
Production Date:	10-Oct-2012



M.W. 284
$C_{19}H_{24}O_2$

PACKAGING: 1.0 mCi/ml (37 MBq/ml) in ethanol, under argon, in a vial which protects the contents from UV light. Shipped on dry ice.

STABILITY AND STORAGE RECOMMENDATIONS: When methyltrienolone, [17α-methyl-^{3}H]- is stored at -20°C in its original solvent and at its original concentration, the rate of decomposition is initially 2-3% over the first three months from date of purification. Stability is nonlinear and not correlated to isotope half-life. Lot to lot variation may occur.

- This product is very light-sensitive. Care should be taken to minimize its exposure to light.

SPECIFIC ACTIVITY RANGE: 70-87 Ci/mmol (2590-3219 GBq/mmol)

RADIOCHEMICAL PURITY: This product was initially found to be greater than 97% when determined by the following methods. The rate of decomposition can accelerate. It is advisable to check purity prior to use:

High pressure liquid chromatography on a Zorbax ODS column using the following mobile phase:
acetonitrile : water, (4:6)

Thin layer chromatography on silica gel using the following solvent system:
toluene : ethyl acetate, (4:1).

QUALITY CONTROL: The radiochemical purity of methyltrienolone, [17α-methyl-^{3}H]- is checked at appropriate intervals using the first listed chromatography method.
*Manufactured by PerkinElmer™ Life and Analytical Sciences under licensed agreement of Roussel UCLAF.

HAZARD INFORMATION: WARNING: This product contains a chemical known to the state of California to cause cancer.

PerkinElmer, Inc.
549 Albany Street
Boston, MA 02118 USA
P: (800) 762-4000 or (+1) 203-925-4602
www.perkinelmer.com/nenradiochemicals
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Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.080
ChemTask No. CHEM11786
CAS No. 27503-81-7

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

[redacted]
Program Information Coordinator

ENSULIZOLE

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted] 09-05-12
Task Leader Date

Approved by:

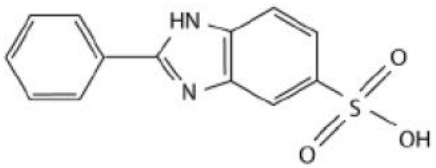
[redacted] 09/05/12
Reshan Fernando, Ph.D. Date
Principal Investigator

Submitted to:

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

ENSULIZOLE

CAS No.: 27503-81-7	Study Lab: (Investigator): ILS ([redacted]
RTI Chemical ID Code: N60	Lot No. (Vendor): 05117JE(Aldrich)
ChemTask No.: CHEM11786	Vendor Purity: 99.9% (by HPLC, Aldrich COA)
RTI Log Nos. (Amt. Received): Analytical: 082010-C-15 (~50 g) Reference: 082010-C-05 (~5 g)	Receipt Date: Aug 20, 2010 (Bulk receipt and reference)
Program Supported: TOX	Receipt Condition: No damage noted
Analysis Dates: May 11, 15 and 24, 2012	Submitter: [redacted] (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.	MOL. FORMULA
	274.30	$C_{13}H_{10}N_2O_3S$

EXECUTIVE SUMMARY

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

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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

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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
1026	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.

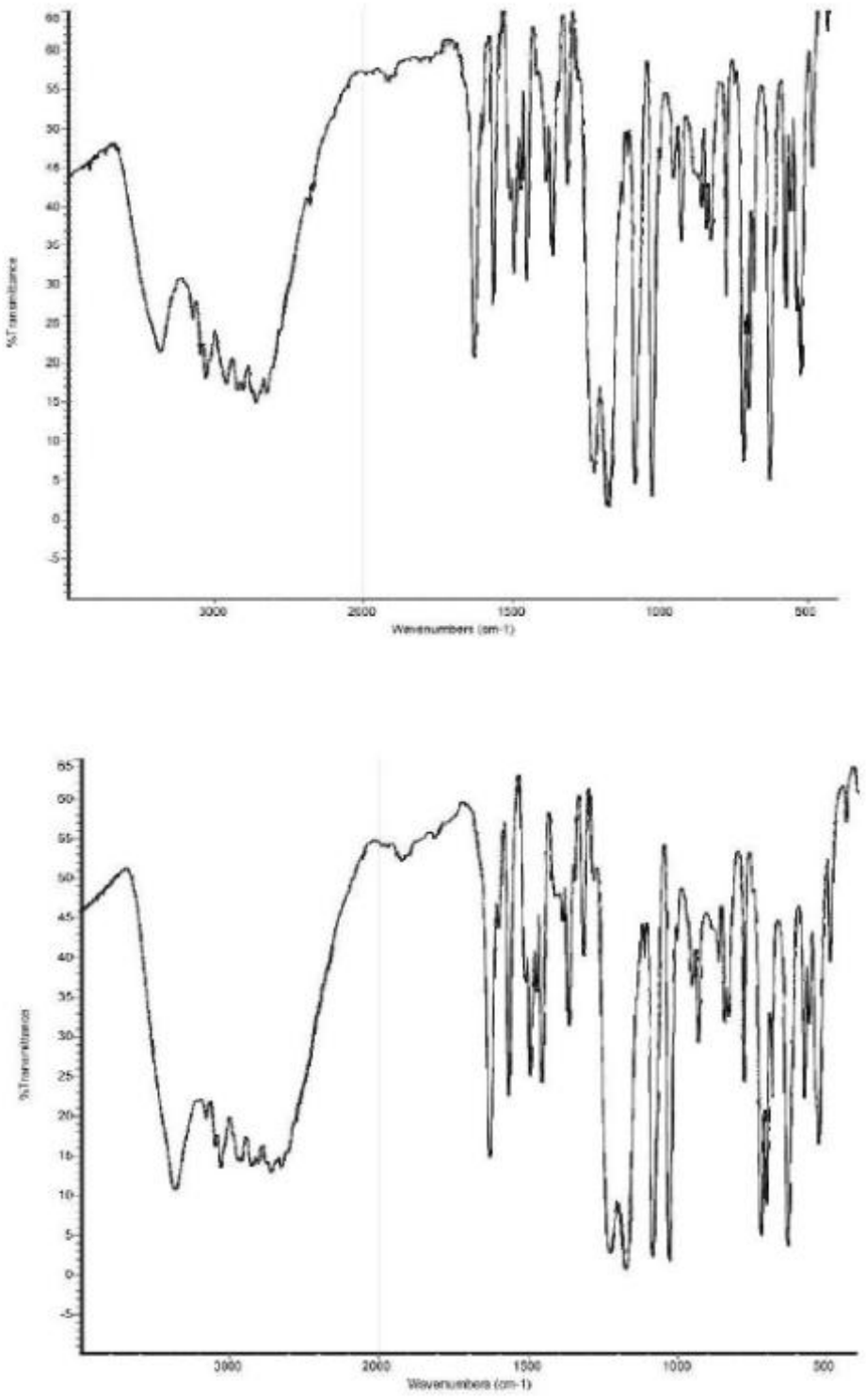


Figure 1: Infrared Spectrum of Ensulizole Frozen Reference (top spectrum) and Bulk Sample (bottom spectrum)

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 5 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.

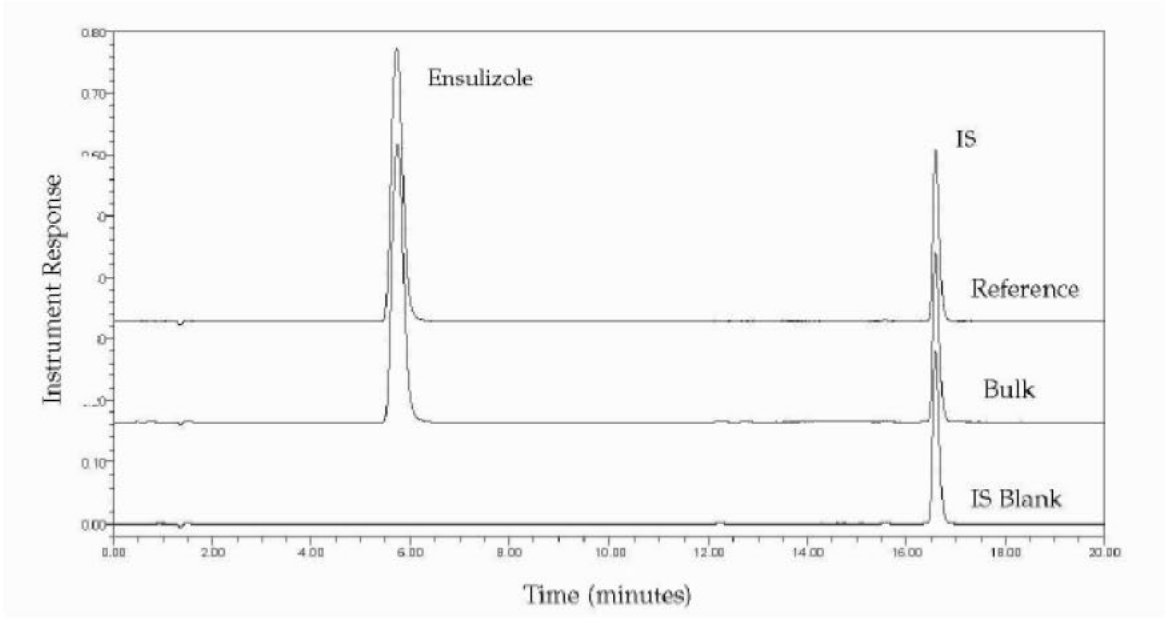


Figure 2: Example Liquid Chromatograms of Ensulizole Reference and Bulk Sample, and a Blank

5.0 REFERENCE

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

6.0 ACKNOWLEDGMENTS

Personnel contributing to this task: [redacted]

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:

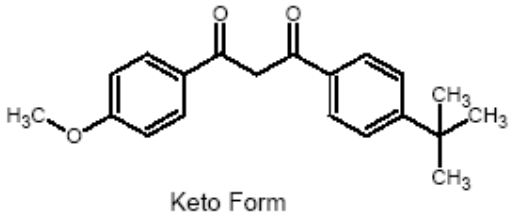
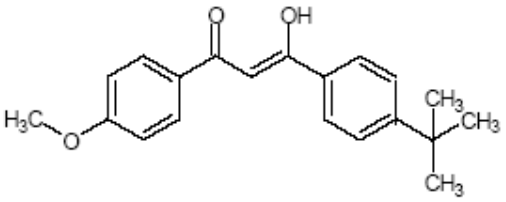
[redacted]
Group Leader

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. CHEM10985 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:

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

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

[redacted]
Program Information Coordinator

HOMOSALATE

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted]
Task Leader

09-05-12
Date

Approved by:

[redacted]
Reshan Fernando, Ph.D.
Principal Investigator

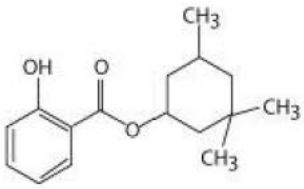
09/05/12
Date

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	$C_{16}H_{22}O_3$

EXECUTIVE SUMMARY

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

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.

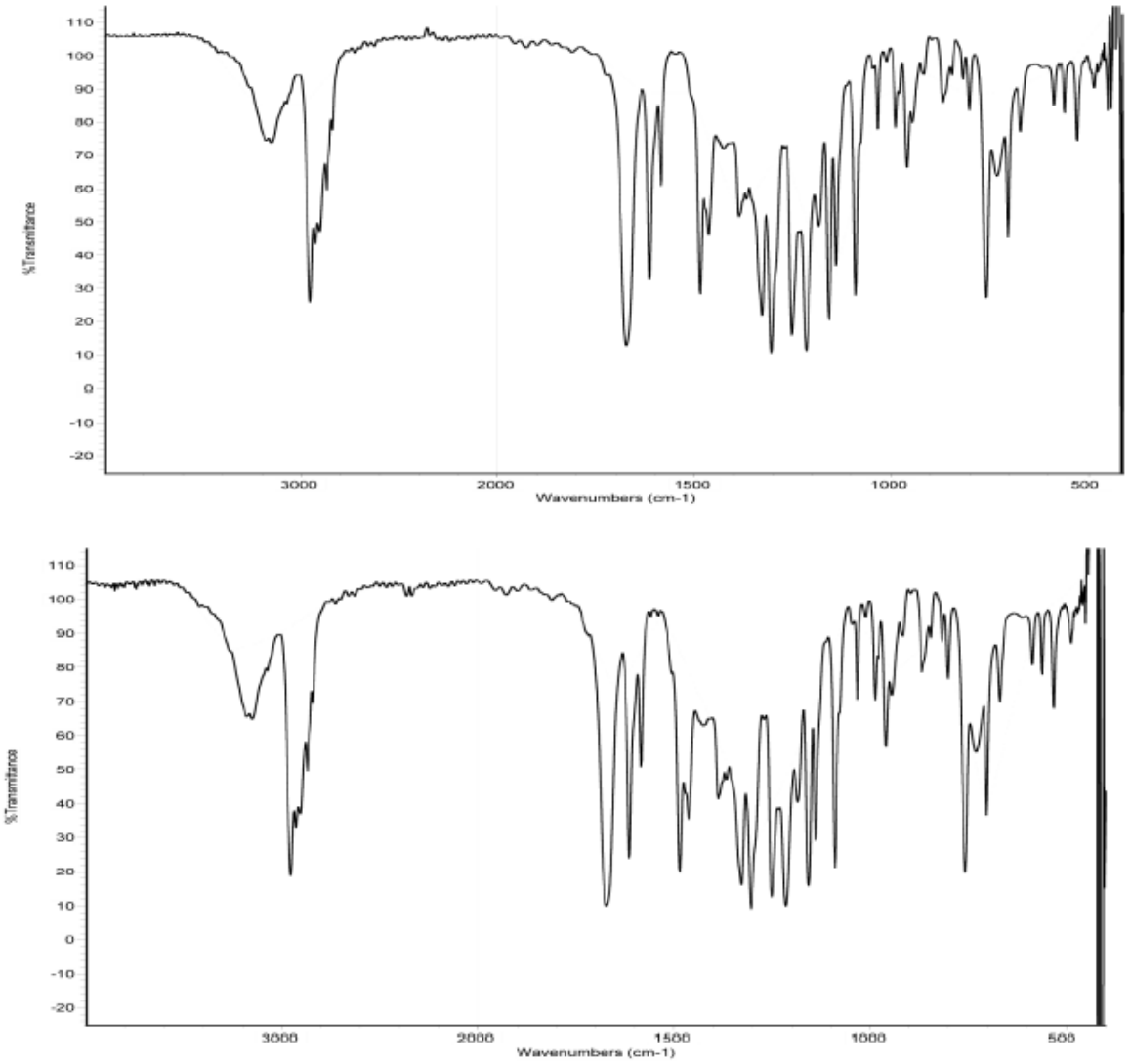

Figure 1: Infrared Spectrum of Homosalate Bulk (top spectrum)
and Frozen Reference (bottom spectrum)

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	≥ 250,000 plates	2,460,486	Pass
Precision (%RSD)	≤ 5% (n=6)	0.2	Pass
Resolution	≥ 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[a] (%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.

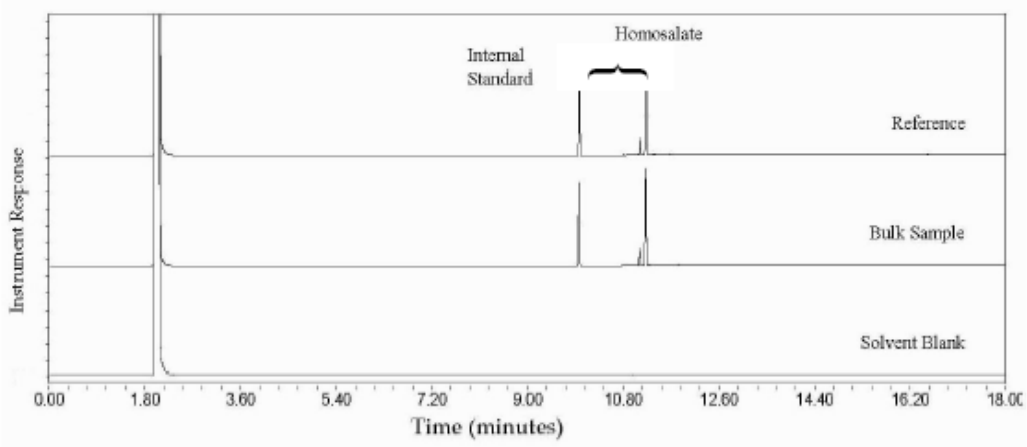

Figure 2: Example Gas Chromatograms of Homosalate Reference and Bulk Sample, and a Blank

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
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Telephone 919.541.6730 or 919.541.5975 • Fax 919.485.2650 • www.rti.org
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Analytical Chemistry Services for the NTP
NIH Contract No. HHSN273201100003C
RTI Project 0212839.200.003.081
ChemTask No. CHEM11787
CAS No. 21245-02-3

This pdf is an exact duplicate of the original approved report

[redacted]
Program Information Coordinator

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

CHEMICAL REANALYSIS

September 5, 2012

Prepared by:

[redacted] 09-05-12
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)

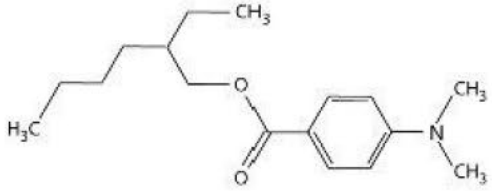
Receipt Date: Aug 20, 2010 (Bulk)

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

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: 0212839.200.003.061

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	05/15/12	05/22/12
Data & Report Audit	08/20/12	08/20/12

Prepared by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

Reviewed by:

[redacted]
Quality Assurance Specialist

9/5/12
Date

turning knowledge into practice

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Figures

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.

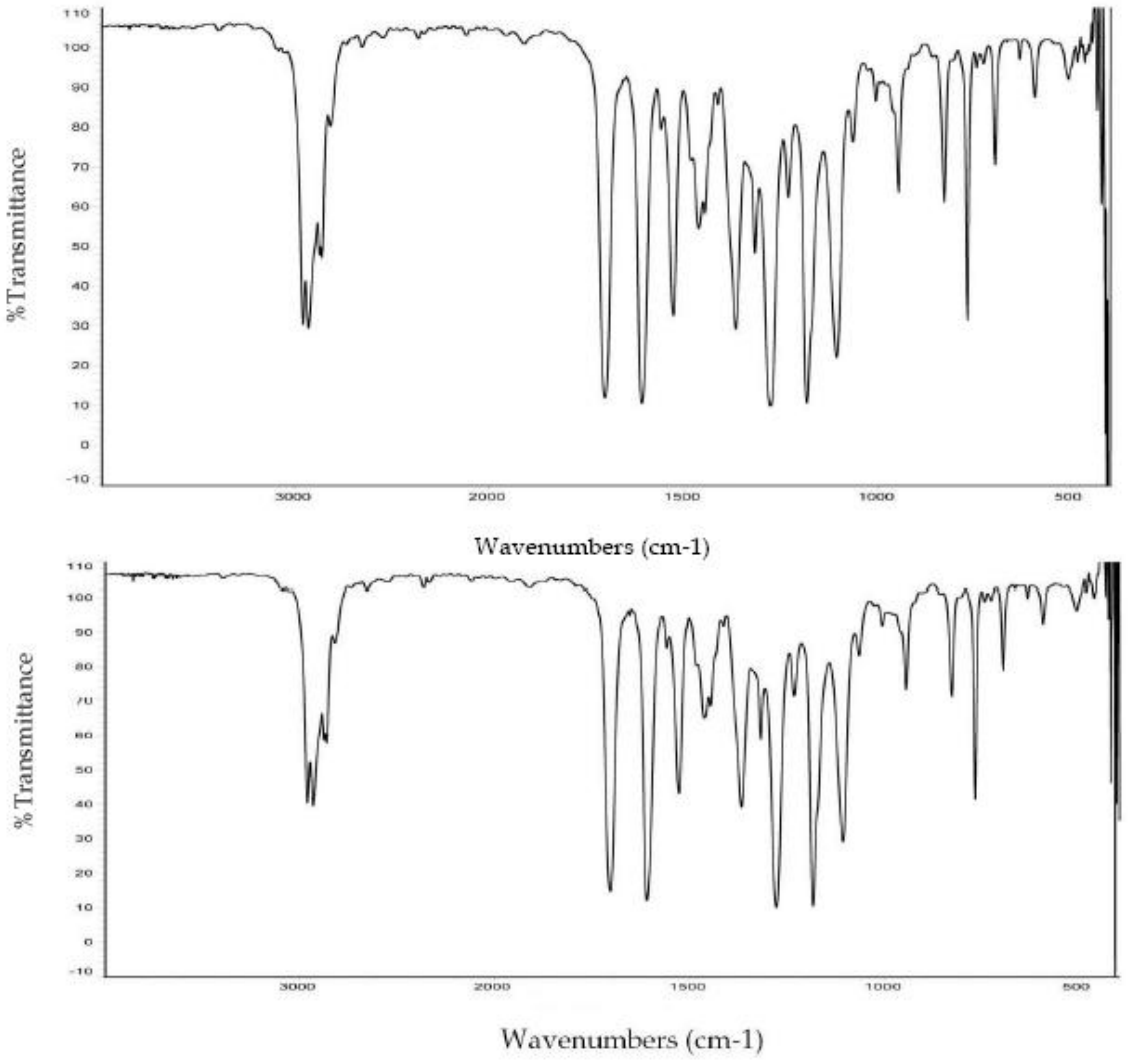

Figure 1: Infrared Spectrum of Padimate O Bulk (top spectrum) and Frozen Reference (bottom spectrum)

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.

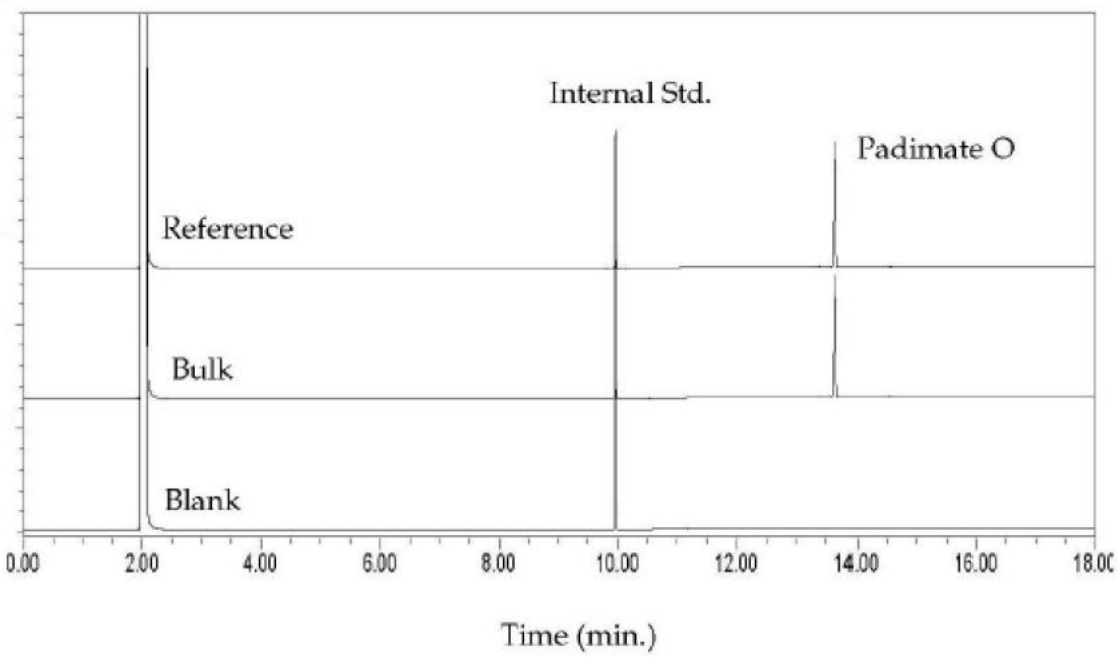

Figure 2: Example Gas Chromatograms of Padimate O Reference and Bulk Sample, and an IS Blank

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 5 Protocol and Protocol Amendment

CeeTox®
In vitro models to predict toxicity

PROTOCOL

Androgen Receptor Binding (Rat Prostate Cytosol)

Data Requirements: *OPPTS 890.1150*

Study Number:
9070-100794ARB

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]

E-mail: [redacted]

Study Monitor: [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:** January 23, 2013 (date subject to change; actual experimental start date to be provided in final report)

***Proposed Experimental Termination Date:** February 22, 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.:
Facsimile No.:
E-mail:

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

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Signatures

[redacted]
Chief, Toxicology Branch
National Toxicology Program, NIEHS
Date 1/18/13

[redacted]
Contract Office Technical Representative
National Toxicology Program, NIEHS
Date 1/15/13

[redacted]
Integrated Laboratory Systems, Inc.
Study Monitor
Date 15Jan2013

[redacted]
Study Director
Date 16 Jan 2013

(1) Actual page is 6 of 19. Pagination changed due to format issues. [redacted] 16Jan13

(2) unknown mark from Sponsor. [redacted] 16Jan13

1. Title of Study

Androgen Receptor Binding (Rat Prostate Cytosol)

2. Purpose of Study

The objective of this study is to evaluate four test substances for the ability to compete with [^{3}H] ligand for binding androgen receptors (ARs) in rat ventral prostate tissue homogenate. The endpoint is the decays per minute (DPM) of the radioligand.

The results of this screen are intended to be used in conjunction with results from other Tier 1 in vitro and in vivo screening assays (OCSPP 890 test guideline series) that constitute the full screening battery under the Endocrine Disruptor Screening Program (EDSP). Results of the Tier 1 screening battery, along with other scientifically relevant information, are to be used in a weight-of-evidence assessment leading to the determination of a substance's potential to interact with the endocrine system. The Tier 1 battery is intended for screening purposes only and should not be used for endocrine classification or risk assessment.

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. The data and the draft final report will be reviewed by the Quality Assurance Unit of CeeTox in accordance with CeeTox standard operating procedures (SOPs).

5. Regulatory Citations

Endocrine Disruptor Screening Program Test Guidelines. *OPPTS 890.1150: Androgen Receptor Binding (Rat Prostate Cytosol).* EPA 640-C-09-003. October, 2009.

6. Test Facility

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

7. Experimental Design

The androgen receptor binding 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 systems. This assay is intended to identify the ability of test compounds to interact with the androgen receptors (ARs) isolated from

Sprague-Dawley rat prostates. In this assay, the test materials and the controls are incubated with rat prostate cytosol (containing the ARs) and radiolabeled R1881 (competitor) for approximately 16-20 hours at approximately 4°C. The amount of bound radiolabeled R1881 is assessed using a scintillation counter to determine the decays per minute (DPM), and specific binding is then determined. A complete concentration response curves for the positive control R1881, the weak positive control (wPC) dexamethasone, and if applicable, the test materials, will be generated each time the binding assay is performed.

8. Justification of the Test System

As per the guideline (OPPTS 890.1150) prostate glands from castrated Sprague-Dawley male rats (60 to 90 days of age at time of kill) will be used to prepare the cytosol. The cytosol will be prepared and then deemed acceptable, per EPA guideline and CeeTox SOP for use in this study. Bias is not a factor in this test system. The test system (cytosol) will be identified by the isolation date. Cytosol preparation and saturation binding data will be included in the appendices of the final report.

9. Identification of the Test System

Prostate glands from castrated Sprague-Dawley male rats will be used to prepare the cytosol. The prostate glands will be purchased from an outside vendor. The cytosol will be prepared, and deemed acceptable, per EPA guideline and CeeTox SOP for use in this study. The test system (cytosol) will be identified by the isolation date. Cytosol preparation information, saturation binding data and any other information deemed necessary will be included in the appendices of the final report.

10. Test & Control Substance(s)

10.1 Test Substance

A certificate of analysis for the test substances will be provided by the sponsor and will be stored in the study data and appended to the study report. Confirmation of the identity of the test substance, 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.

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:	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:	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:	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%

10.2 Preparation of Test Substance

The stock test substances (approximately 100 mM, depending on solubility or other factors) will be formulated in dimethyl sulfoxide (DMSO) or appropriate solvent. Fresh 30X dilutions of the stock solution will be prepared in Low-Salt TEDG (Tris, EDTA, DTT, Glycerol) Buffer on the day of use such that the target concentration of test substance can be achieved by the addition of approximately 10 µL of the dilution to an approximately 300 µL total assay volume. Dose concentrations of test and control substances will not be verified using analytical methods.

Serial Dilutions of Test Substances

Serial dilutions of test substances will be prepared in Low-Salt TEDG assay buffer, to yield the final concentrations indicated in Table 2, unless solubility limits the top concentration tested.

Table 1. Test Substance Dilution Procedure

Tube #	Volume of stock to add for diluted concentration	Volume of TEDG Assay Buffer	Total volume of diluted test substance	Diluted test substance concentration	*Final test substance concentration in AR assay tube
TS1	Use 300 µl of stock test substance (100 mM)	700 µl	1 ml	3×10^{-2} M	1×10^{-3} M
TS2	Use 100 µl of dilution TS1 (50 mM)	900 µl	1 ml	3×10^{-3} M	1×10^{-4} M
TS3	Use 100 µl of dilution TS2 (5 mM)	900 µl	1 ml	3×10^{-4} M	1×10^{-5} M
TS4	Use 100 µl of dilution TS3 (500 µM)	900 µl	1 ml	3×10^{-5} M	1×10^{-6} M
TS5	Use 100 µl of dilution TS4 (50 µM)	900 µl	1 ml	3×10^{-6} M	1×10^{-7} M
TS6	Use 100 µl of dilution TS5 (5 µM)	900 µl	1 ml	3×10^{-7} M	1×10^{-8} M
TS7	Use 100 µl of dilution TS6 (500 nM)	900 µl	1 ml	3×10^{-8} M	1×10^{-9} M
TS8	Use 100 µl of dilution TS7 (50 nM)	900 µl	1 ml	3×10^{-9} M	1×10^{-10} M

*Final concentration of test substance in assay tube when 10 µl of diluted concentration is used in a total volume of 300 µl.

10.3 Reference Substances

When testing substances for their ability to bind to the androgen receptor (AR), a solvent control, positive control (standards) and weak positive control will be included in each experiment. One set of standards is needed in each run on a given day.

A standard curve using R1881 (CAS 965-93-5; positive control) will be included to allow for an assessment of variability in the conduct of the experiment across time. The concentration range for R1881 will be 1 x 10^{-11} to 1 x 10^{-6} M. The supplier, catalog number, lot number and purity will be included in the final report.

Table 2. Example of Dilution Procedure for Reference Standard R1881

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of R1881	Diluted R1881 concentration	Final R1881 concentration in AR assay tube
N/A	Use 100 µl of stock R1881 (10 mM)	900 µl	1 ml	1 x 10^{-3} M	N/A
NSB	Use 30 µl of stock R1881 (1 mM)	970 µl	1 ml	3 x 10^{-5} M	1 x 10^{-6}
S2	Use 100 µl of dilution NSB (30 µM)	900 µl	1 ml	3 x 10^{-6} M	1 x 10^{-7}
S3	Use 100 µl of dilution S2 (3 µM)	900 µl	1 ml	3 x 10^{-7} M	1 x 10^{-8}
S4	Use 100 µl of dilution S3 (300 nM)	900 µl	1 ml	3 x 10^{-8} M	1 x 10^{-9}
S5	Use 100 µl of dilution S4 (30 nM)	900 µl	1 ml	3 x 10^{-9} M	1 x 10^{-10}
S6	Use 100 µl of dilution S5 (3 nM)	900 µl	1 ml	3 x 10^{-10} M	1 x 10^{-11}

The weak positive substance (dexamethasone; CAS 50-02-2) will be included to demonstrate the sensitivity of each experiment and to allow an assessment of variability of the conduct of the experiment across time. The concentration range for dexamethasone will be 1 x 10^{-10} to 1 x 10^{-3} M. The supplier, catalog number, lot number and purity will be included in the final report.

Table 3. Example of Dilution Procedure for Dexamethasone

Tube #	Volume of stock to add for diluted concentration	Volume of solvent to add	Total volume of diluted dexamethasone	Diluted dexamethasone concentration	Final dexamethasone concentration in AR assay tube
wPC1	Use 300 µl of stock wPC (100 mM)	700 µl	1 ml	3 x 10^{-2} M	1 x 10^{-3} M
wPC2	Use 100 µl of dilution wPC1 (50 mM)	900 µl	1 ml	3 x 10^{-3} M	1 x 10^{-4} M
wPC3	Use 100 µl of dilution wPC 2 (5 mM)	900 µl	1 ml	3 x 10^{-4} M	1 x 10^{-5} M
wPC4	Use 100 µl of dilution wPC3 (500 µM)	900 µl	1 ml	3 x 10^{-5} M	1 x 10^{-6} M
wPC5	Use 100 µl of dilution wPC4 (50 µM)	900 µl	1 ml	3 x 10^{-6} M	1 x 10^{-7} M
wPC6	Use 100 µl of dilution wPC5 (5 µM)	900 µl	1 ml	3 x 10^{-7} M	1 x 10^{-8} M
wPC7	Use 100 µl of dilution wPC6 (500 nM)	900 µl	1 ml	3 x 10^{-8} M	1 x 10^{-9} M
wPC8	Use 100 µl of dilution TS7 (50 nM)	900 µl	1 ml	3 x 10^{-9} M	1 x 10^{-10} M

The Radioactive Ligand ([^{3}H]–R1881) supplier, catalog number and batch number will be included in the final report. The specific activity (SA) and date for which that SA was certified by the supplier will be included along with the concentration as received from the supplier (Ci/mmol) and the concentrations tested (nM).

11. Stock Solution Preparation

Preparation of Stock Solutions for making Low-Salt TEDG (Tris, EDTA, DTT, Glycerol) Buffer

- 200 mM EDTA Stock Solution: For example, 7.444g disodium EDTA will be added to 100 ml purified water. This solution will be stored at approximately 4°C.
- 1M Sodium Molybdate Stock Solution: For example, 10 ml purified water will be added to 2.419g sodium molybdate. The buffer will be stored at approximately 4°C.
- 1M Tris Buffer: For example, 147.24g Tris-HCl and 8.0g Tris base will be added to 800 ml purified H_2O. The final volume will be brought to 1 Liter. The buffer will be refrigerated to approximately 4°C and then pH to approximately 7.4. The buffer will be stored at approximately 4°C.

Preparation of Low-Salt TEDG Buffer (pH approximately 7.4)

- For example to make 100 ml of low-salt TEDG buffer, the following will be added in order:
- 87.15 ml purified water
- 1.0 ml 1M Tris
- 10.0 ml glycerol
- 100 µl 1M sodium molybdate
- 750 µl 200 mM EDTA
- 0.5 ml Calbiochem Protein Inhibitor Cocktail, Set III, EDTA Free (with PMSF) or equivalent
- 15.4 mg DTT (will be added immediately before use, see below)
- The pH of the final solution will be checked to make sure it is approximately 7.4 at approximately 4°C (the solution will be adjusted with HCl (approximately 1M) or NaOH (approximately 1N) as necessary).
- 15.4 mg DTT will be added directly to 100 ml low-salt TEDG buffer the morning of the receptor isolation (final concentration = 1 mM DTT).

Preparation of 60% hydroxyapatite (HAP) slurry

For example to prepare 1L of 50 mM Tris Buffer:

- 50 ml of 1M Tris will be added to 950 ml purified water. This solution will be stored at approximately 4°C. The pH of the final solution will be checked to make sure it is approximately 7.4 at approximately 4°C (the solution will be adjusted as necessary).
- BIO-RAD HT-Gel (Bio-Rad; Hercules, CA) or equivalent will be shaken until all the hydroxyapatite (HAP) is in suspension. The evening before the receptor extraction, an appropriate volume will be poured into a graduated cylinder, the top will be sealed and placed in the refrigerator for at least 2 hours.
- The phosphate buffer supernatant will be poured off, and the volume brought to 100 ml with 50 mM Tris. The HAP will be suspended by sealing the top and inverting several times and left to settle. The HAP will be washed two more times with fresh 50 mM Tris buffer, as above. After the last wash, the HAP is left to settle overnight at 4°C.
- The next morning the 50 mM Tris is removed and the fully settled HAP is resuspended in enough 50 mM Tris to make the final solution 60% slurry.
- This will be stored at approximately 4°C until ready for use in the extraction.

Preparation of [^{3}H]-R1881 Stock Solutions

- For example the original stock of [^{3}H]–R1881 will be diluted to 0.1 µM (i.e., 1 X 10-^{7} M) by pipetting approximately 1 µl of the stock solution for every specific activity unit (Ci/mmol) and diluting this to approximately 10.0 ml with ethanol. The [^{3}H]–R1881 stock solution and dilutions will be stored at approximately -20°C.
- A 1 x 10^{-8} M stock of [^{3}H]–R1881 will be prepared by making a 10-fold dilution of the 1 x 10^{-7} M stock.
- A copy of the Certificate of Analysis for [^{3}H]–R1881 will be maintained with the study records.

Preparation of 100X Radioinert R1881 Solutions

- A 5 mM solution of R1881 will be prepared in DMSO. For example, 5.00 mg of radioinert R1881 will be weighed in a tared amber vial and 3.516 ml solvent added. The 5 mM stock 1:500 will be diluted in the same solvent to get the 10 µM stock. The R1881 stock solution and dilutions will be stored at approximately -20°C.
- The 1 µM radioinert R1881 stock will be prepared by diluting the 10 µM stock 1:10 in an amber vial. This will be the 1 µM radioinert R1881 stock.
- The 0.1 µM radioinert R1881 stock will be prepared by pipetting the 1 µM stock 1:10 in an amber vial. This will be the 0.1 µM radioinert R1881 stock.

Preparation of Triamcinolone Acetonide Stock and Working Solutions

- For 600 µM solution, for example 13.04 mg of triamcinolone acetonide will be added to DMSO in a total volume of 50 ml. This will be mixed thoroughly and stored at approximately -20°C.

- The desired amount of 60 µM triamcinolone acetonide working solution will be prepared for the assay by making a 1:10 dilution of the 600 µM stock in DMSO. This will be mixed thoroughly and stored at approximately -20°C.

12. Competitive Radioligand Binding Assay

The competitive binding assay will be performed a minimum of three times. The optimal amount of cytosolic protein added will contain enough receptor to bind no more than 10-15% of the radiolabeled R1881 that has been added to the tube.

Preparation of test substance stock solutions

Test substances will be prepared at 30X the desired final concentration (listed in Table 2). Initial stocks will be prepared in assay buffer at a concentration of approximately 30 mM.

Table 4. Summary of Assay Conditions

		Competitive Binding Assay Protocol
Source of receptor		Rat prostate cytosol
Concentration of radioligand		1 nM
Concentration of receptor		Sufficient to bind 10-15% of radioligand)
Concentration of test substance (as serial dilutions)		100 pM to 1 mM
Temperature		~4°C
Incubation time		16-20 hours
Composition of assay buffer	Tris	10 mM (pH 7.4)
	EDTA	1.5 mM
	Glycerol	10% (v/v)
	Protease Inhibitor	0.5% (v/v)
	DTT	1 mM
	Sodium Molybdate	1 mM

The specific activity (SA) of [^{3}H]-R1881 will be adjusted for decay over time. The SA will be calculated on the day of the assay using the following equation:

$$SA_{adjusted} = SA * e^{-Kdecay*Time}$$

$SA_{adjusted}/SA$ = Fraction Isotope Remaining (FIR)

Where:

SA is the specific activity on the packaging date (both SA and the packaging date are printed on the stock bottle from the manufacturer).

K_{decay} is the decay constant for tritium (equal to 1.54×10^{-4}/day)

Time = number of days since the printed date on the stock bottle from the manufacturer

The [^{3}H]-R1881 will be diluted with TEDG + PI buffer so that each assay tube contains 1 nM final concentration of [^{3}H]-R1881 using the following procedure:

The specific activity will be converted from Ci/mmole to nM. If SA = X Ci/mmole, and Y = concentration of radiolabel, then X Ci/mmole will be converted to nM and the SA activity adjusted for decay over time by the following conversion:

(Y mCi/ml / X Ci/mmole) * 1 Ci/1000 mCi * 10^6 nmole/mmole * 1000 ml/L = (Y/X) * 10^6 nM.

A 10 nM diluted stock of the [^{3}H]-R1881 was prepared so that 30 µl in a total volume of 300 µl per assay tube gave a final concentration of 1 nM. The 10 nM [^{3}H]-R1881 was kept on ice until standards, test chemicals, and assay tubes were prepared.

Assay Preparations

12 x 75 mm (or appropriately sized) siliconized glass tubes will be used for the assay. Approximately 30 µl of 10 nM [^{3}H]-R1881 (1 x 10^{-8} M) and approximately 50 µl triamcinolone acetonide (60 µM working solution) will be added to all tubes. For the 3 tubes at the beginning of assay and at the end of assay, 100X inert R1881 (30 µl of 1 µM) will also be added; these are the nonspecific binding tubes. The tubes will be placed in a speed-vac and dried. An aliquot of cytosol will be thawed on ice and diluted to the pre-determined optimal protein concentration.

Individual Tubes

For the assay tubes, approximately 10 µl of each concentration of test chemical or control will be added by pipette, followed by approximately 300 µl of the diluted cytosol. The temperature of the tubes and contents will be kept at approximately 4°C prior to the addition of the cytosol. The assay tubes will be vortexed after additions and incubated at approximately 4°C for approximately 16 to 20 hours on a rotator.

Separation of bound [^{3}H]-R1881 from free [^{3}H]-R1881

Following the approximately 16-20 hour incubation, the AR assay tubes will be removed from the rotator and placed in an ice-water bath. A repeating pipette will be used to add approximately 500 µl of ice cold HAP slurry (60% in 50 mM Tris buffer) to fresh new 12 x 75 mm (or appropriately sized) siliconized assay tubes. Approximately 100 µl of each incubation tube will be transferred to the appropriate labeled tubes containing the HAP. The tubes will be vortexed for approximately 10 seconds at approximately 5 minute intervals for a total of approximately 20 minutes with tubes remaining in the ice-water bath between vortexing. Following the vortexing step, approximately 2 ml of the cold 50 mM Tris buffer will be added, the tubes will be quickly vortexed, and centrifuged at approximately 4°C for approximately 10 minutes at 700 x g. After centrifugation, the supernatant containing the free [^{3}H]-R1881 will immediately be decanted and discarded. The HAP pellet contains the androgen receptor bound [^{3}H]-R1881. Approximately 2 ml of ice-cold 50 mM Tris buffer will be added to each tube and vortexed to resuspend the pellet. The tubes will be centrifuged again at approximately 4°C for approximately 10 minutes at approximately 700 x g. The supernatant will be quickly decanted and discarded. The wash and centrifugation steps will be repeated three more times. After

the final wash, the supernatant will be decanted. The assay tubes will be allowed to drain briefly for approximately 30 seconds.

Extraction and Quantification of [^{3}H]-R1881 bound to AR

Approximately 2 ml of absolute ethanol will be added to each assay tube. The tubes will be allowed to sit at room temperature for approximately 15 to 20 minutes, vortexing for approximately 10 seconds at approximately 5-minute intervals. The assay tubes will be centrifuged for approximately 10 minutes at approximately 700 x g. The supernatant will be decanted into a 20 ml scintillation vial containing an appropriate amount of scintillation cocktail (Perkin Elmer Opti-Fluor, cat# 6013199, or equivalent). The vial will be capped and shaken. The vials will be placed in a scintillation counter (Perkin Elmer Tri-Carb 2910TR Liquid Scintillation Analyzer Model B2910, or equivalent) and each vial will be counted for at least one minute with quench correction for determination of DPMs per vial.

13. Solubility/Precipitation Assay

The limit of test substance solubility will be determined by laser based light scattering. The test substance will be prepared in the TEDG buffer alone (no cytosol) at the final exposure concentrations and added to wells of a 96-well plate. The samples will be assessed using a NEPHELOstar nephelometer (BMG LabTech, Ortenberg, Germany), or equivalent.

14. Competitive Binding Data Analyses

Estimating the IC_{50}

An AR competitive binding assay measures the binding of a single concentration of [^{3}H]-R1881 in the presence of increasing concentrations of a test substance. The competitive binding curve is plotted as specific [^{3}H]-R1881 binding (as a percent of total binding) versus the concentration (log_{10} units) of the competitor. The concentration of the test substance that inhibits 50% of the maximum specific [^{3}H]-R1881 binding is the IC_{50} value. Estimates of IC_{50} values are determined using XLfit (Guildford, Surrey, UK).

Calculation of RBA

The relative binding affinity (RBA) for each competitor will be calculated by dividing the IC_{50} for R1881 by the IC_{50} of the competitor and expressing as a percent.

$$\% \text{ RBA} = \frac{IC_{50}\ \text{R1881}}{IC_{50}\ \text{Test Substance}} \times 100$$

Competitive Binding Performance Criteria

The competitive binding assay is functioning correctly if all of the following criteria have been met:

Increasing concentrations of unlabeled R1881 displace [^{3}H]-R1881 from the receptor in a manner consistent with one-site competitive binding. Specifically, the curve fitted to the radioinert R1881 data points using non-linear regression descend from 90% to 10% over approximately an 81-fold increase in the concentration of radioinert R1881.

Ligand depletion is minimal.

The parameter values (top, bottom, and slope) for the standard curve (R1881) and the weak positive control are within the tolerance bounds provided in Table 6.

The solvent control substance does not alter the sensitivity or reliability of the assay.

Table 5. Suggested Upper and Lower Limits for Parameters in Competitive Binding Assay Curves for the Standards (Radioinert R1881 and Dexamethasone)

Substance	Parameter	Lower Limit	Upper Limit
Standard Curve	Slope	-1.2	-0.8
	Top (%)	82	114
	Bottom (%)	-2.0	+2.0
Weak Positive	Slope	-1.4	-0.6
	Top (%)	87	106
	Bottom (%)	-12	+12

For all test substances, it is recommended that the top of the curve fall within 80-115% binding.

15. Proposed Statistical Methods

For each of the three valid independent runs of the competitive binding assays, the following statistics will be assessed; mean specific binding (%), standard deviation (SD), standard error of the mean (SEM), percent coefficient of variation (% CV), residuals, squared residuals, and the $Log_e(S_{yx})$ (ie. Log_e(residual standard deviation)) using XLfit (Guildford, Surrey, UK).

16. Classification Criteria

The classification of a test substance as a binder or non-binder is made on the basis of the average results of three runs. The data interpretation criteria are presented in Table 7.

Table 6. Data Interpretation Criteria

	Criteria	Classification
Data fit 4-parameter nonlinear regression model	Average curve across all runs crosses 50%*	Binder
	Average lowest portion of the curves across all runs is between 50 and 75% activity**	Equivocal
	Average lowest portion of curves across all runs is greater than 75% activity**	Non-Binder
Data do not fit the model	---	

*Ordinarily, a binding curve will fall from 90% to 10% over 2 log units with a slope near -1. If the curve falls outside the range for the weak positive control (-0.6 to -1.4), the run will be classified as equivocal. Unusually steep curves may be a sign that the protein is being denatured or that solubility problems are being encountered.

**If the test substance is not soluble above 10^{-6} M and the binding curve does not cross 50%, the substance is judged to be untestable. If the curve is steeper than -2.0 the result is considered to be equivocal.

17. Final Study Reports

The data to be reported will be determined per Standard Operating Procedure (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, data analysis and interpretation and classification of the test substances.

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. Data 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.

Protocol Amendment 1

Study Number: 9070-100794ARB

Title of Study to be Amended: Androgen Receptor Binding (Rat Prostate Cytosol)

Reason for Amendment to Protocol: This amendment to correct two sections of the protocol. First, section 10.2 Preparation of Test Substance (page 10) states that:

"The stock test substances (approximately 100 mM, depending on solubility or other factors) will be formulated in dimethyl sulfoxide (DMSO) or appropriate solvent. Fresh 30X dilutions of the stock solution will be prepared in Low-Salt TEDG (Tris, EDTA, DTT, Glycerol) Buffer on the day of use such that the target concentration of test substance can be achieved by the addition of approximately 10 µL of the dilution to an approximately 300 µL total assay volume. Dose concentrations of test and control substances will not be verified using analytical methods."

This section will now read as follows (changes in bold red):

"The stock test substances (approximately 100 mM, depending on solubility or other factors) will be formulated in dimethyl sulfoxide (DMSO) or appropriate solvent. Fresh 30X dilutions of the stock solution will be prepared in **DMSO** on the day of use such that the target concentration of test substance can be achieved by the addition of approximately 10 µL of the dilution to an approximately 300 µL total assay volume. Dose concentrations of test and control substances will not be verified using analytical methods."

This amendment is also to correct the type of assay tube used in the study. The protocol states, in Section 12, page 15 under the subsection Assay Preparations and the subsection Separation of bound [^{3}H]-R1881 from free [^{3}H]-R1881 that "12 x 75 mm (or appropriately sized) siliconized glass tubes" will be used.

The OPPTS 890.1150 guideline does not state that the glass tubes need to be siliconized for the ARB assay. Therefore, non-siliconized tubes will be used throughout the assay.

Signatures

[redacted] 2/18/13
Chief, Toxicology Branch Date
National Toxicology Program, NIEHS

[redacted] 2/13/13
Contract Office Technical Representative Date
National Toxicology Program, NIEHS

[redacted] 12 Feb 2013
Integrated Laboratory Systems, Inc Date
Study Monitor

[redacted] 13 Feb 2013
Study Director Date
CeeTox, Inc.