

FINAL REPORT

Study Title

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY
Using AE, AC, and AF**

Authors

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Study Completion Date

January 31, 2006

Performing Laboratory

Institute for In Vitro Sciences, Inc.
21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

Study Number

05AF53-AF55.350056

Laboratory Project Number

4207

STATEMENT OF NO DATA CONFIDENTIALITY CLAIMS

No claim of confidentiality is made for any information contained in this study on the basis of its falling within the scope of FIFRA section 10 (d) (1) (A), (B), or (C).

Company:

Company Agent: _____

Date: _____

Title

Signature: _____

STATEMENT OF COMPLIANCE

The Bovine Corneal Opacity and Permeability Assay With Two Time Exposures and Optional Histology of the test substances, AE, AC, and AF, were conducted in compliance with the U.S. FDA Good Laboratory Practice Regulations as published in 21 CFR 58, the U.S. EPA GLP Standards 40 CFR 160 and 40 CFR 792, and the OECD Principles of Good Laboratory Practice in all material aspects with the following exceptions:

The identity, strength, purity and composition or other characteristics to define the test substances have not been determined by the testing facility.

Study Director _____ Date _____
John W. Harbell, Ph.D.

Submitter: _____ Date _____

Sponsor's Representative: _____ Date _____

QUALITY ASSURANCE STATEMENT

Study Title: Bovine Corneal Opacity and Permeability Assay with Two Time Exposures and Optional Histology

Study Number: 05AF53-AF55.350056.350056

Study Director: John Harbell, Ph.D.

This study has been divided into a series of in-process phases. Using a random sampling approach, Quality Assurance monitors each of these phases over a series of studies. Procedures, documentation, equipment records, etc., are examined in order to assure that the study is performed in accordance with the U.S. FDA Good Laboratory Practice Regulations (21 CFR 58), the U.S. EPA GLP Standards (40 CFR 792 and 40 CFR 160) and the OECD Principles of Good Laboratory Practice and to assure that the study is conducted according to the protocol and relevant Standard Operating Procedures.

The following are the inspection dates, phases inspected and report dates of QA inspections of this study:

Phase Inspected	Audit Date(s)	Reported to Study Director	Reported to Management
Protocol and Initial Paperwork	21-Sep-05	21-Sep-05	26-Sep-05
Initial Opacity Measurement	29-Sep-05 03-Oct-05	04-Oct-05 04-Oct-05	04-Oct-05 04-Oct-05
Final Report and Data	19&20-Jan-06	20-Jan-06	31-Jan-06

This report describes the methods and procedures used in the study and the reported results accurately reflect the raw data of the study.

Amanda K. Ulrey, RQAP-GLP
Quality Assurance

Date

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**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

Initiation Date: September 21, 2005

Completion Date: January 31, 2006

Sponsor:

Sponsor's Representative:

Testing Facility: Institute for In Vitro Sciences, Inc.
21 Firstfield Road, Suite 220
Gaithersburg, MD 20878

Archive Location: Institute for In Vitro Sciences, Inc.
Gaithersburg, MD 20878

Study Director: _____
John W. Harbell, Ph.D. Date

Lead Biologist: Valerie Deoudes, B.S.

Laboratory Management: Greg Mun, B.A.

TEST SUBSTANCE RECEIPT

IIVS Test Substance Number	Sponsor's Designation	Physical Description	Receipt Date	Storage Conditions*
05AF53	AE	clear red non-viscous liquid	9/1/05	room temperature
05AF54	AC	clear purple non-viscous liquid	9/1/05	room temperature
05AF55	AF	clear light pink non-viscous liquid	9/1/05	room temperature

* - Protected from exposure to light

**BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY WITH
TWO TIME EXPOSURES AND OPTIONAL HISTOLOGY**

SUMMARY

The ocular irritancy of AE, AC, and AF were evaluated in the Bovine Corneal Opacity and Permeability (BCOP) Assay. Positive and negative control materials were tested concurrently in the study. Each treatment condition was applied to three corneas. The corneas were exposed to the test substances for periods of 3 and 10 minutes followed by a post-exposure incubation of 2 hours. After determination of the final opacity measurements and assessment of the penetration of fluorescein, each cornea was fixed for possible histological evaluation. Histological evaluation was not performed on the corneas.

**Summary Table
BCOP Results of the Test Substances**

Assay Date	IIVS Test Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD ₄₉₀ Value	<i>In Vitro</i> Score	pH
10/3/05	05AF53	AE	Neat	3 minutes	28.7	0.606	37.8	11.5
				10 minutes	41.7	1.668	66.7	
	05AF54	AC	Neat	3 minutes	85.0	1.068	101.0	0.5
				10 minutes	113.3	1.432	134.8	
	05AF55	AF	Neat	3 minutes	2.3	0.013	2.5	2.0
				10 minutes	9.3	0.020	9.6	

The response of the positive control, neat ethanol, was within the normal range and therefore the assay was considered valid according the acceptance criteria of the protocol.

INTRODUCTION

The purpose of this study was to evaluate the potential ocular irritancy of the test substances, AE, AC, and AF, in the Bovine Corneal Opacity and Permeability (BCOP) Assay. The test substances, AE, AC, and AF, were exposed to the corneas using 750 μ L per cornea (normal BCOP exposure method). The positive and negative control materials were exposed to the corneas using 750 μ L per cornea. This study was performed at the Institute for In Vitro Sciences, Inc. (IIVS), 21 Firstfield Road, Suite 220, Gaithersburg, MD, 20878. The protocol was signed by the Study Director on September 21, 2005 and the assay was begun on September 29, 2005. The first trial was invalid because the positive control response was not within the acceptable range. Therefore a second trial was performed on October 3, 2005 and the results of that trial are reported. Histological evaluation was not performed on the corneas.

MATERIALS AND METHODS

Test Substance Preparation

As instructed by the Sponsor, each test substance was administered to the test system without dilution.

Test Substance pH Determination

The pH of each test substance was determined using pH paper (EMD Chemicals Inc./ EM Science). Initially, each test substance was added to 0-14 pH paper with 1.0 pH unit increments to approximate a narrow pH range. Next, each test substance was added to 7.5-14.0 pH paper or 0-6 pH paper with 0.5 pH unit increments, to obtain a more accurate pH value. The pH values obtained from the narrower range pH paper are presented in Table 1.

Assay Controls

The positive control used in this study was neat ethanol (Pharmco). The negative control used in this study was sterile, deionized water (Quality Biological).

Bovine Eyes

Bovine eyes were obtained from a local abattoir as a by-product from freshly slaughtered animals (J.W. TREUTH & SONS, Inc., Baltimore, MD). The eyes were excised and then placed in Hanks' Balanced Salt Solution, containing Penicillin/Streptomycin (HBSS), and transported to the laboratory on ice packs. Immediately upon receipt of the eyes into the laboratory, preparation of the corneas was initiated.

Preparation of Corneas

The eyes were grossly examined for damage and those exhibiting defects were discarded. The tissue surrounding the eyeball was carefully pulled away and the cornea was excised such that a 2 to 3 mm rim of sclera was present around the cornea. The isolated corneas were then stored in a petri dish containing HBSS until they were mounted in a corneal holder. The corneas were mounted in the holders with the endothelial side against the O-ring of the posterior chamber. The anterior chamber was then positioned on top of the cornea and the screws were tightened. Starting with the posterior chamber, the two chambers were then filled with Minimum Essential Medium (EMEM) without phenol red, containing 1% fetal bovine serum and 2 mM L-glutamine (Complete MEM). Each corneal holder was uniquely identified with a number written in permanent marker, on both the anterior and posterior chambers. The corneal holders were incubated at $32 \pm 1^\circ\text{C}$ for a minimum of 1 hour.

Bovine Corneal Opacity and Permeability Assay

After a minimum of 1 hour of incubation, the corneas were removed from the incubator. The medium was removed from both chambers and replaced with fresh Complete MEM. The initial opacity was determined for each cornea using a Spectro Designs OP-KIT opacitometer. Three corneas, whose initial opacity readings were close to the median opacity for all the

corneas, were selected as the negative control corneas. The treatment of each cornea was identified with the test substance number written in permanent marker on colored tape, affixed to each holder. The medium was then removed from the anterior chamber and replaced with the test substance, positive control, or negative control.

Method for Testing Liquid or Surfactant Materials

The test substances were tested in two definitive assays. The results of the first definitive assay (performed on September 29, 2005) were considered invalid because the positive control produced an *in vitro* score that did not fall within the acceptable range. Only the results of the second definitive assay (performed on October 3, 2005) with acceptable positive control results were considered valid and are presented in this report.

The liquid test substances, AE, AC, and AF, were tested neat. An aliquot of 750 μL of the test substance, positive control, or negative control was introduced into the anterior chamber while slightly rotating the holder to ensure uniform distribution over the cornea. One group of three corneas was incubated in the presence of each test substance at $32 \pm 1^\circ\text{C}$ for 3 minutes. A second group of three corneas was incubated in the presence of each test substance at $32 \pm 1^\circ\text{C}$ for 10 minutes. A group of three corneas was incubated in the presence of the positive control at $32 \pm 1^\circ\text{C}$ for 10 minutes. Another group of 3 corneas was incubated in the presence of the negative control at $32 \pm 1^\circ\text{C}$ for 10 minutes. After the 3 and 10-minute exposure times, the control or test substance treatments were removed. The epithelial side of the corneas was washed at least three times with Complete MEM (containing phenol red) to ensure total removal of the control or test substances. The corneas were then given a final rinse with Complete MEM (without phenol red). The anterior chamber was refilled with fresh Complete MEM (without phenol red) and an opacity measurement was performed. The corneas were returned to the incubator for approximately 2 hours after which a final measure of opacity was obtained.

After the final opacity measurement was performed, the medium was removed from both chambers of the holder. The posterior chamber was filled with fresh Complete MEM and 1 mL of a 4 mg/mL fluorescein solution was added to the anterior chamber. The corneas were then incubated in a horizontal position (anterior side up) for approximately 90 minutes at $32 \pm 1^\circ\text{C}$. At the end of the 90-minute incubation period, the medium was removed from the posterior chamber and placed into tubes numbered corresponding to chamber number. Aliquots of 360 μL from the numbered tubes were placed into their designated wells on a 96-well plate. The optical density at 490 nm (OD_{490}) was determined using a Molecular Devices Vmax kinetic microplate reader. If the OD_{490} value of a control or test substance sample was 1.500 or above, a 1:5 dilution of the sample was prepared in Complete MEM (to bring the OD_{490} value within the linear range of the platereader). A 360 μL sample of each 1:5 dilution was transferred to its specified well on the 96-well plate. The plate was read again and the final reading was saved to a designated print file.

Fixation of Corneas

After the medium was removed for the permeability determination, each cornea was carefully separated from its corneal holder and transferred to an individual pre-labeled tissue cassette containing a biopsy sponge. The endothelial surface of each cornea was placed on the sponge to protect it. The cassettes were placed in 10% neutral buffered formalin to fix the

corneal tissue for at least 24 hours. The fixed corneas will be stored up to one year.

Histological Evaluation

As instructed by the Sponsor, a histological evaluation was not performed.

Presentation of Data

Opacity Measurement: The change in opacity for each cornea (including the negative control corneas) was calculated by subtracting the initial opacity reading from the final opacity reading. These values were then corrected by subtracting from each the average change in opacity observed for the negative control corneas. The mean opacity value of each treatment group was calculated by averaging the corrected opacity values of each cornea for that treatment condition.

Permeability Measurement: The mean OD₄₉₀ for the blank wells was calculated. The mean blank OD₄₉₀ was then subtracted from the raw OD₄₉₀ of each well (corrected OD₄₉₀). Any dilutions that were made to bring the OD₄₉₀ readings into the linear range of the platereader (OD₄₉₀ should be less than 1.500), had each diluted OD₄₉₀ reading multiplied by the dilution factor. The final corrected OD₄₉₀ of the test substances and the positive control was then calculated by subtracting the average corrected OD₄₉₀ of the negative control corneas from the corrected OD₄₉₀ value of each treated cornea:

$$\text{Final Corrected OD}_{490} = (\text{raw OD}_{490} - \text{mean blank OD}_{490}) - \text{average corrected negative control OD}_{490}$$

The mean OD₄₉₀ value of each treatment group was calculated by averaging the final corrected OD₄₉₀ values of the treated corneas for that treatment condition.

The following formula was used to determine the *in vitro* score:

$$\text{In Vitro Score} = \text{Mean Opacity Value} + (15 \times \text{Mean OD}_{490} \text{ Value})$$

Criteria for Determination of a Valid Test

The BCOP assay was accepted when the positive control (ethanol) caused an *in vitro* score that fell within two standard deviations of the historical mean.

RESULTS AND DISCUSSION

Bovine Corneal Opacity and Permeability Assay

Table 1 summarizes the opacity, permeability, and *in vitro* score for each test substance. Table 2 summarizes the opacity, permeability, and *in vitro* score for the positive control. Since the results of the positive control fell within two standard deviations of the historical mean (within a range of 40.1 to 65.0), the assay was considered valid. The opacity and permeability data for the individual corneas may be found in Appendix B.

The following classification system was established by Sina et al.¹ based on studies with a wide range of test materials. While this classification system provides a good initial guide to interpretation of these *in vitro* data, these specific ranges may not be applicable to all classes of materials.

In Vitro Score:

from 0 to 25	= mild irritant
from 25.1 to 55	= moderate irritant
from 55.1 and above	= severe irritant

Table 1
BCOP Results of the Test Substances

Assay Date	IIVS Test Substance Number	Sponsor's Designation	Conc.	Exposure Time	Mean Opacity Value	Mean OD ₄₉₀ Value	<i>In Vitro</i> Score	pH
10/3/05	05AF53	AE	Neat	3 minutes	28.7	0.606	37.8	11.5
				10 minutes	41.7	1.668	66.7	
	05AF54	AC	Neat	3 minutes	85.0	1.068	101.0	0.5
				10 minutes	113.3	1.432	134.8	
	05AF55	AF	Neat	3 minutes	2.3	0.013	2.5	2.0
				10 minutes	9.3	0.020	9.6	

¹Sina, J.F., Galer, D.M., Sussman, R.G., Gautheron, P.D., Sargent, E.V., Leong, B., Shah, P.V., Curren, R.D., and Miller, K. (1995) A collaborative evaluation of seven alternatives to the Draize eye irritation test using pharmaceutical intermediates. **Fundamental and Applied Toxicology** 26:20-31.

Table 2
BCOP Results of the Positive Control

Assay Date	Positive Control	Exposure Time	Mean Opacity Value	Mean OD₄₉₀ Value	<i>In Vitro</i> Score
10/3/05	Ethanol	10 minutes	31.3	1.325	51.2

APPENDIX A

APPENDIX B

BOVINE CORNEAL OPACITY AND PERMEABILITY ASSAY

OPACITY SCORE

<u>TA #</u>	<u>CORNEA #</u>	<u>INITIAL</u>	<u>FINAL</u>	<u>CHANGE</u>	<u>CORRECTED</u>	<u>AVG</u>	<u>STDEV</u>
05AF53	13	4	37	33	32.0		
Neat	14	2	30	28	27.0		
3 minutes	15	2	30	28	27.0	28.7	2.9
05AF53	17	3	47	44	43.0		
Neat	18	3	46	43	42.0		
10 minutes	20	3	44	41	40.0	41.7	1.5
05AF54	21	3	80	77	76.0		
Neat	22	3	97	94	93.0		
3 minutes	23	4	91	87	86.0	85.0	8.5
05AF54	25	4	118	114	113.0		
Neat	26	5	156	151	150.0		
10 minutes	27	3	81	78	77.0	113.3	36.5
05AF55	28	4	8	4	3.0		
Neat	32	4	10	6	5.0		
3 minutes	33	4	4	0	-1.0	2.3	3.1
05AF55	34	4	18	14	13.0		
Neat	37	3	12	9	8.0		
10 minutes	38	4	12	8	7.0	9.3	3.2
Neg. Control	1	3	4	1	NA		
Sterile, DI water	2	3	4	1	NA		
10 minutes	3	3	4	1	NA	1.0	
Pos. Control	4	3	34	31	30.0		
Ethanol	8	3	36	33	32.0		
10 minutes	12	4	37	33	32.0	31.3	1.2
	*6	1					
	*10	5					
	*40	4					
	*41	4					
	*42	3					
	*43	5					
	*44	3					
	*45	2					
	*46	4					
	*47	6					
	*48	4					
	*49	4					
	*50	4					
	*51	5					
	*52	3					

Initial corneal opacity average: 4

* - Corneas not used in this assay, but used to find initial opacity average.
 NA - Not Applicable

PERMEABILITY SCORE

**Neg. Control
Sterile, DI water
10 minutes**

Cornea #	OD490
1	0.006
2	0.003
3	0.004

Avg.	0.004

**05AF53
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
13	0.349	1	0.345
14	0.793	1	0.789
15	0.689	1	0.685

Avg. =			0.606
STDEV =			0.232

**05AF54
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
21	0.330	5	1.646
22	0.599	1	0.595
23	0.968	1	0.964

Avg. =			1.068
STDEV =			0.533

**05AF55
Neat
3 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
28	0.015	1	0.011
32	0.028	1	0.024
33	0.009	1	0.005

Avg. =			0.013
STDEV =			0.010

**Pos. Control
Ethanol
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
4	1.367	1	1.363
8	1.257	1	1.253
12	1.365	1	1.361

Avg. =			1.325
STDEV =			0.063

**05AF53
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
17	1.459	1	1.455
18	0.426	5	2.126
20	1.428	1	1.424

Avg. =			1.668
STDEV =			0.397

**05AF54
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
25	1.293	1	1.289
26	0.389	5	1.941
27	1.071	1	1.067

Avg. =			1.432
STDEV =			0.454

**05AF55
Neat
10 minutes**

Cornea #	OD490	Dilution Factor	Corrected OD490
34	0.017	1	0.013
37	0.025	1	0.021
38	0.030	1	0.026

Avg. =			0.020
STDEV =			0.007

Performed on October 3, 2005

Study No. 05AF53-AF55.350056 B2

IN VITRO SCORE**In Vitro Score = Mean Opacity Value + (15 x Mean OD490)**

Test Substance	Concentration	Exposure Period	Mean Opacity	Mean OD490	In vitro Score
05AF53	Neat	3 minutes	28.7	0.606	37.8
05AF53	Neat	10 minutes	41.7	1.668	66.7
05AF54	Neat	3 minutes	85.0	1.068	101.0
05AF54	Neat	10 minutes	113.3	1.432	134.8
05AF55	Neat	3 minutes	2.3	0.013	2.5
05AF55	Neat	10 minutes	9.3	0.020	9.6
Ethanol	Neat	10 minutes	31.3	1.325	51.2