The Utilization of the EpiOcular™ Human Tissue Model to Assess and Compare the Irritation Potential of Multiple Surfactant Systems Used in Shampoos and Facial Cleansers

Vavilikolanu, P.[1], Lazaro, C.[1], Mun, G.[2], Hilberer, A.[2], Hyder, M.[2], Raabe, H.[2], and Curren, R.[2]
[1]Alberto-Culver Company, Melrose Park, IL, USA
[2]Institute for In Vitro Sciences, Inc., Gaithersburg, MD, USA

Presented at the 47th Annual Society of Toxicology Meeting
Seattle, WA
March 17-20, 2008

ABSTRACT

Assuring the safety of cosmetics and personal care products without testing in animals is a primary goal for Alberto-Culver Company. In addition, the Seventh Amendment to the Cosmetics Directive requires that after 2009, animal testing cannot be used to assess the eye or skin irritation potential of either cosmetic formulations or ingredients. To address these issues, we have developed an in vitro irritation assessment program to support the ocular safety evaluation of multiple surfactant systems used in shampoos and facial cleansers. This is particularly important as eye irritation is a foreseeable occurrence in the use of these cosmetics and personal care products. The program relies on the results of a topical application of formulations to the surface of a three-dimensional, human cell-derived model of the corneal epithelium (EpiOcular™, MatTek Corp., Ashland, MA, USA) and monitoring time to toxicity. 35 finished products and 15 prototype formulations with a range of multiple surfactant systems have been tested at dilutions of 2% and 10% (w/v in water). Two surfactant reference standards with well established safety profiles in commerce were tested along with these materials at same dilutions of 2% and 10%. The irritation potential of materials was then assessed by comparison to these benchmark materials. At these dilutions, we determined that the irritancy potential for most of the prototype shampoos fell in the mild to no irritation range shown as similar and less cytotoxic responses compared to the Reference materials. The effectiveness of this in vitro test system was evaluated by comparing the in vitro test results with consumer experience information.

INTRODUCTION

Surfactants are regularly used in a wide range of personal care and cosmetic products such as shampoos, facial cleansers, body washes, etc. In a quest to assure safety, preferably through a non-animal testing program and to meet the requirements of the European Union's Seventh Amendment to the Cosmetics Directive, Alberto-Culver Company developed an in vitro program for the evaluation of ocular safety for surfactant systems used in shampoos, facial cleansers, body washes, etc. The EpiOcular™ model assay was determined to be a suitable assay system to predict the potential ocular irritancy of a set of chemicals used in cosmetics (Kay, 1962; McCain, 2002), and a related protocol has been used in a broader evaluation of surfactants and surfactant mixtures (Blazka, 2003). We chose the human corneal epithelial constructs over other in vitro models in order to reduce the variance from the human response that might be associated with using ex vivo corneal cells from other species (e.g. bovine or porcine). While we expect some level of irritation, we want to be able to use this method as a basis for making commercialization decisions for prototype products as well. In this way we can assess the utility of our program in identifying possible issues before product reaches the market.

We present the data generated in the Alberto-Culver program in collaboration with The Institute for In Vitro Sciences (IIVS), Inc. for ocular safety. We present the results of 35 different finished products and 15 prototypes (shampoos, facial cleansers, and body washes) formulated with a range of single and multiple surfactant systems at dilutions of 2% and 10% (w/v in water). Potential safety of the materials was assessed by comparison of the ET_{50} results (an EpiOcular™ assay endpoint) to two benchmarks with a well established history of consumer acceptance. Many of the products tested so far have ET_{50} values greater than 4 hours at the 2% dilution, suggesting that they fall in the mild to no irritation range. The effectiveness of the in vitro model has been assessed by comparing the in vitro results with consumer experience information.

MATERIALS AND METHODS

Media and reagents

- Assay Medium and the EpiOcular™ Tissue Constructs (OCL-200) - MatTek Corporation (Ashland, MA).
- Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine (MTT Addition Medium)
- 10X stock solution of 3-(4, 5 - dimethylthiazol-2-yl) - 2, 5 - diphenyltetrazolium bromide (MTT) and the sterile, deionized water - Quality Biological (Gaithersburg, MD) (10 mg/mL MTT in PBS)
- Dulbecco's Phosphate buffered Saline without Ca^{++} and Mg^{++} - (pH 7.0+0.5) MatTek Corporation (Ashland, MA) or equivalent.

Test system

The EpiOcular™ Human Cell Construct model (OCL-200) is a three-dimensional non-keratinized tissue construct composed of normal human derived epidermal keratinocytes used to model the human corneal epithelium (**Figure 1**). The EpiOcular™ model offers the advantage of applying test materials topically to the tissue test system, so that water insoluble materials as well as water soluble materials can be tested. The ocular irritation potential of a test material is based on the time it takes to reduce tissue viability by 50% (ET_{50}) as measured by the tissue's ability to reduce MTT. The irritation potential of a test material is inversely related to the ET_{50}. The MTT conversion assay measures the nicotinamide adenine dinucleotide phosphate [NAD(P)H]-dependent microsomal enzyme reduction of MTT (and to a lesser extent, the succinate dehydrogenase reduction of MTT) to a blue formazan precipitate following exposure to the test material for various exposure times (Berridge, 1996).

Test material preparation and administration

Test materials were shampoos, facial cleansers, SPF cleansers and body washes of a viscous or semi-viscous nature (**Table 1**). To better reflect various normal exposure scenarios, test materials were tested at a 2% (w/v) (20 mg/mL) dilution in water. Test materials were also tested at an alternate dilution of 10% (w/v) (100 mg/mL), which is considered as a conservative dilution and commonly used as the dilution factor in both the in vitro and in vivo safety assessment programs.

Treatment of cultures

A schematic of the assay procedure is given in **Figure 2**. One hundred µL of the diluted test material were applied directly on the tissue using a positive displacement pipette so as to cover the upper surface. Two tissues were used for each test article exposure time. A general prediction model for this type of protocol using cosmetics and personal care products was proposed by McCain, 2002 and MatTek and is shown in **Table 2**.

Controls, Reference Materials and Killed Controls

To ensure that the EpiOcular™ tissues met the quality control standards, a positive control was tested in each assay using 100 µl of 0.3% Triton®-X-100 for 15 and 45 minutes, in duplicate cultures. The results of an assay are considered valid only when the positive control ET_{50} result falls within the established acceptable range generated from the IIVS positive control historical results. The negative control (100 µL of sterile, deionized water) was tested at exposure times of 15 minutes and 4 hours to assure the proper tissue condition and to provide an appropriate 100% viability response in the range of exposure times up to 4 hours.

As an added assessment tool, two materials with similar chemistry and well established safety assessment data were selected and tested in parallel with other test materials as Reference Materials (**Table 3** Products #49 and #50). These Reference Materials were tested at same dilutions (2% and 10%) as the test materials.

A false negative result can occur in this type of assay if the test material itself is able to reduce MTT and if the test material remains on the tissue after the rinsing step (**Figure 2**). For test materials with positive MTT reduction responses, a killed control experiment was performed using freeze-killed tissues to determine whether residual test material was acting to directly reduce the MTT. To test for residual test material reduction, killed tissues were treated with the test material in the normal fashion for at least the shortest and longest exposure times. At least one killed control treated with sterile deionized water (negative killed control) was tested for the longest negative control exposure time since a small amount of MTT reduction is expected from the residual NADH and associated enzymes within the killed tissue. If little or no MTT reduction was observed in the test article-treated killed control, the MTT reduction observed in the test article-treated viable tissue was ascribed to the viable cells. If there was appreciable MTT reduction in the treated killed control (relative to the amount in the treated viable tissue), additional calculations were performed to account for the chemical reduction. The test materials used in this study showed an insignificant effect in the killed control experiments.

MTT tissue viability assessment

Following exposure, the test material was removed by rinsing the cultures in DPBS and then soaking the cultures in 5 ml of the assay medium for 10-20 minutes. The cultures were rinsed, blotted and then placed in wells containing MTT solution (1.0 mg/mL) for 3 hours. Afterwards, the excess MTT was blotted on paper towels and reduced MTT was extracted from the tissue in 2 ml isopropanol. The absorbance of the extraction solution was measured at 550nm (OD_{550}) and was corrected by subtracting the mean OD of the blank control from all wells. Percent viability was calculated using the following equation:

% of Control = (Corrected OD_{550} of Test article Exposure Time / Corrected OD_{550} of Negative Control) X 100

The ET_{50} was interpolated from each plot of % viability vs. exposure time. When all of the exposure time points showed greater than 50% survival, the ET_{50} was listed as greater than the longest exposure time.

FIGURE 1. Cross section of EpiOcular™ tissue. The EpiOcular™ tissue construct models the top epithelial layer and not the stromal and endothelial layers of the cornea.

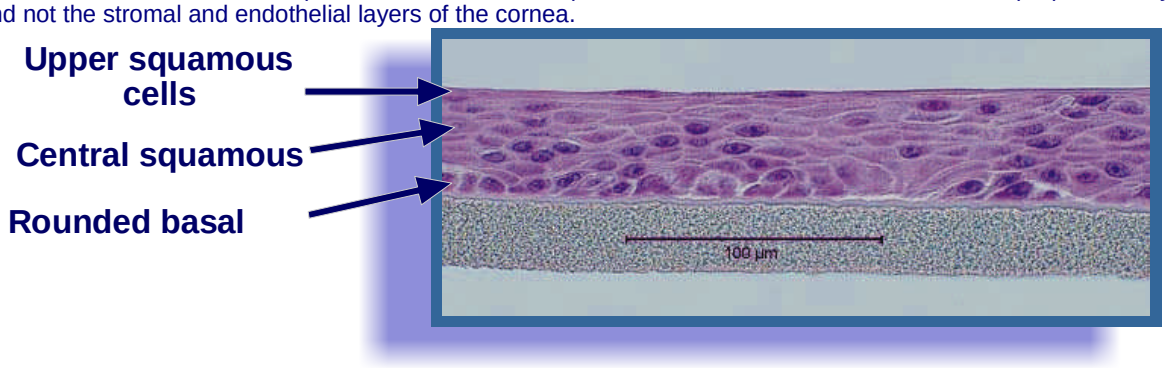


Table 1. Summary of surfactant systems used in the test battery.

Surfactant INCI Name	Description
Sodium Lauryl Sulfoacetate	Mono 1
Sodium Laureth Sulfate, Cocamidopropyl Betaine	Dual 1
Sodium Lauryl Sulfate, Cocamidopropyl Betaine	Dual 2
Decyl Glucoside, Cocamidopropyl Betaine	Dual 3
Sodium Lauryl Sulfoacetate, Cocamide MEA	Dual 4
Sodium Laureth Sulfate, Cocamide DEA, Cocamidopropyl Betaine	TriBlend 1
Ammonium Laureth Sulfate, Ammonium Lauryl Sulfate, Cocamidopropyl Betaine	TriBlend 2
Sodium Lauryl Sulfate, Sodium Laureth Sulfate, Cocamidopropyl Betaine	TriBlend 3
Ammonium Laureth Sulfate, Ammonium Lauryl Sulfate, Cocamide MEA	TriBlend 4
Sodium Laureth Sulfate, Sodium Lauryl Sulfoacetate, Cocamidopropyl Betaine	TriBlend 5
Potassium Laureth Phosphate, Sodium Methyl 2Sulfolaurate, Disodium 2Sulfolaurate, Cocamide MEA	Quattro 1

DEA = diethanolamine; INCI = International Nomenclature of Cosmetic Ingredients; MEA = monoethanolamine.

FIGURE 2. General protocol steps for the EpiOcular™ assay.

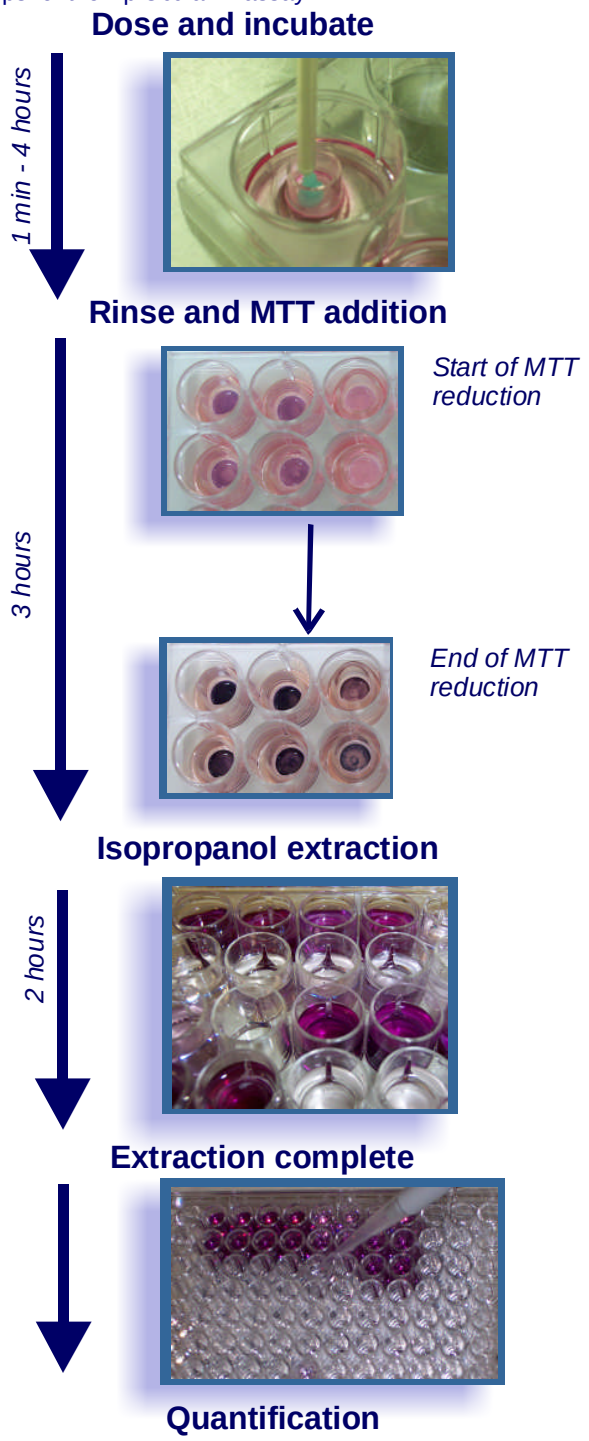


Table 2. A prediction model for personal care and cosmetic products based on in vivo results from Kay, 1962 and ET-50 results from McCain, 2002 and MatTek irritation category based on Draize score and ET_{50}. PEG, polyethylene glycol.

Draize score	Irritancy classification	Example	EpiOcular ET_{50} (minutes)
0-15	Non-irritating, minimal	PEG-75 lanolin, Tween 20	>256-26.5
15.1-25	Mild	3% Sodium dodecyl sulfate	<26.5-11.7
25.1-50	Moderate	5% Triton®X-100	<11.7-3.45
50.1-110	Severe, extreme	5% Benzalkonium chloride	<3.45

Table 3. Test results in consideration of product category, surfactant system and consumer experience.

Product	Product Category	Surfactant	Dilution (w/v)	ET_{50} hrs	Consumer Experience
1	Facial Cleanser	Mono 1	2%	>4	0
2	Facial Cleanser	Mono 1	2%	2.4	NA
3	Facial Cleanser	Mono 1	2%	>4	NA
4	Shampoo	Dual 1	2%	1.9	0
5	Shampoo	Dual 1	2%	2.1	0
6	Shampoo	Dual 1	2%	>4	0
7	Shampoo	Dual 1	2%	3.4	0
8	Facial Cleanser	Dual 2	2%	>4	NA
9	Facial Cleanser	Dual 2	2%	>4	NA
10	Facial Cleanser	Dual 3	2%	3.6	0
11	Facial Cleanser	Dual 3	10%	1.8	0
12	Body Wash	Dual 4	2%	>4	NA
13	Body Wash	Dual 4	2%	>4	NA
14	Body Wash	Dual 4	10%	0.68	NA
15	Body Wash	Dual 4	10%	0.68	NA
16	Body Wash	Dual 4	10%	0.73	NA
17	Body Wash	Dual 4	10%	0.7	NA
18	Body wash	Dual 4	10%	0.82	0
19	Shampoo	TriBlend 1	2%	>4	0
20	Shampoo	TriBlend 1	2%	>4	0
21	Shampoo	TriBlend 1	2%	>4	0
22	Shampoo	TriBlend 1	2%	>4	0
23	Shampoo	TriBlend 1	2%	>4	0
24	Shampoo	TriBlend 1	2%	>4	0
25	Shampoo	TriBlend 1	10%	0.8	NA
26	Shampoo	TriBlend 2	2%	3.1	+
27	Shampoo	TriBlend 2	2%	3.5	+
28	Shampoo	TriBlend 2	2%	3.2	+
29	Shampoo	TriBlend 2	2%	2.6	+
30	Shampoo	Tribelnd 3	2%	3.3	+
31	Shampoo	Tribelnd 3	2%	3.5	+
32	Shampoo	Triblend 3	2%	2.2	0
33	Shampoo	Triblend 3	2%	2.4	0
34	Shampoo	Triblend 3	2%	1.8	0
35	Shampoo	Triblend 3	2%	1.8	0
36	Shampoo	Triblend 3	2%	2.1	0
37	Shampoo	Triblend 3	2%	1.8	+
38	Shampoo	Triblend 3	2%	1.8	+
39	Shampoo	Triblend 3	2%	2.2	+
40	Shampoo	Triblend 3	2%	2.7	0
41	Shampoo	Triblend 3	2%	>4	0
42	Shampoo	TriBlend 5	10%	0.51	0
43	Facial Cleanser	TriBlend 4	2%	3	NA
44	Facial Cleanser	TriBlend 4	2%	3.8	NA
45	Facial Cleanser	Quattro 1	2%	>4	NA
46	Facial Cleanser	Quattro 1	2%	>4	NA
47	Facial Cleanser	Quattro 1	10%	2.6	0
48	Facial Cleanser	Quattro 1	10%	2.7	0
49-Reference material #1	Shampoo	Triblend 3	2%	>4	+
50-Reference material #2	Shampoo	Triblend 3	2%	1.85	+

Note:
Consumer Adverse Experience (over at least a one year period): **0** = no complaints; **+** = a few complaints, but not considered significant; **++** = average complaints, **+++** = potential product problem, and **++++** = significant adverse complaints. **NA** applies to prototype products that have not been commercialized.

ET_{50}: when all of the test material exposure times showed greater than 50% viability, the ET_{50} value was presented as greater than the longest test material exposure time (i.e., "> 4 hours").

RESULTS

Figures 3, 4, 5 and **6** are examples of the exposure time response curves generated in a typical EpiOcular™ assay conducted for this program. The positive control ET_{50} values were in the acceptable range for each assay conducted for this study. An example of the positive control with an ET_{50} of 36.9 minutes is shown in **Figure 3**. Two Reference materials were tested in this program. The Reference material #1 (Product #49) was tested at a dilution of 2% (**Figure 4**) and the ET_{50} was greater than 4 hours thus falling in the mild to no irritation range. The Reference material #2 (Product #50) was tested at both 2% and 10% (**Figure 5**) and had an ET_{50} of 1.85 hours at 2% and an expectidely shorter ET_{50} of 0.23 hours at 10%. **Figure 6A, 6B** shows the exposure time response for Products #23 and #37, respectively (see **Table 3**) when tested at 2%. Product #23 had an ET_{50} greater than 4 hours (**Figure 6a**) and comparable to Reference material #1 (**Figure 4**). According to the consumer experience analysis (**Table 3**), no complaints were received for this product whereas few complaints were registered for Product #37, which had an ET_{50} of 1.8 hours (**Figure 6B**). The in vitro data correlate well with the consumer experience information thus making the EpiOcular™ test system very useful for deciding whether a product moves forward from prototype to market.

Figure 3. Positive control (0.3% Triton -X-100) exposure time response.

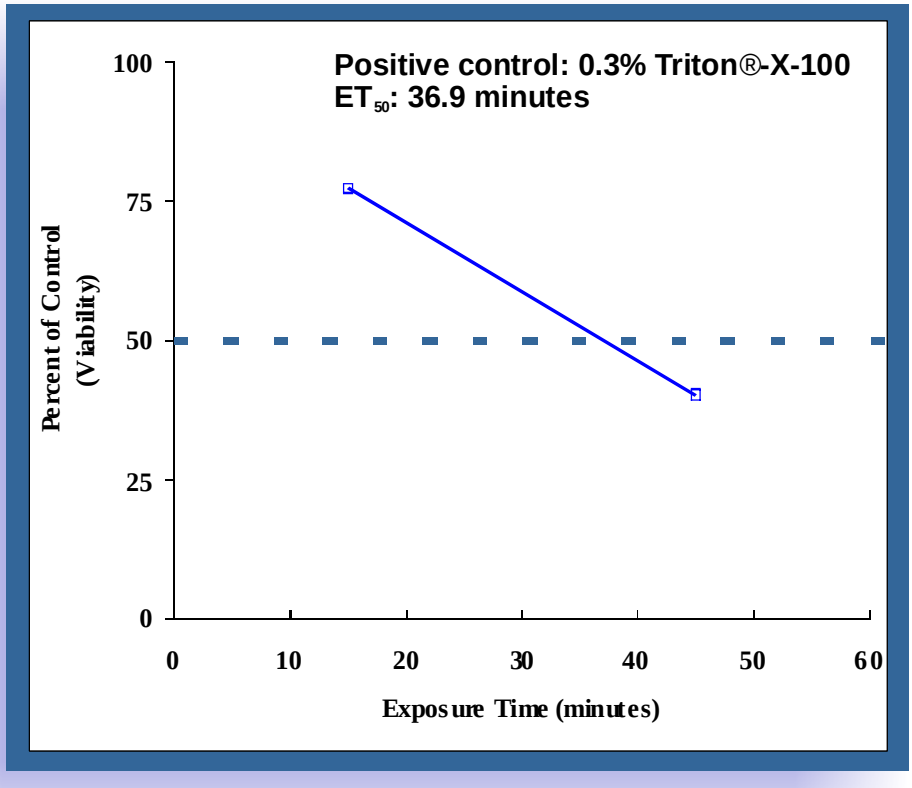


Figure 4. Reference material #1 (Product #49) (see **Table 2**, tested at 2%) exposure time response.

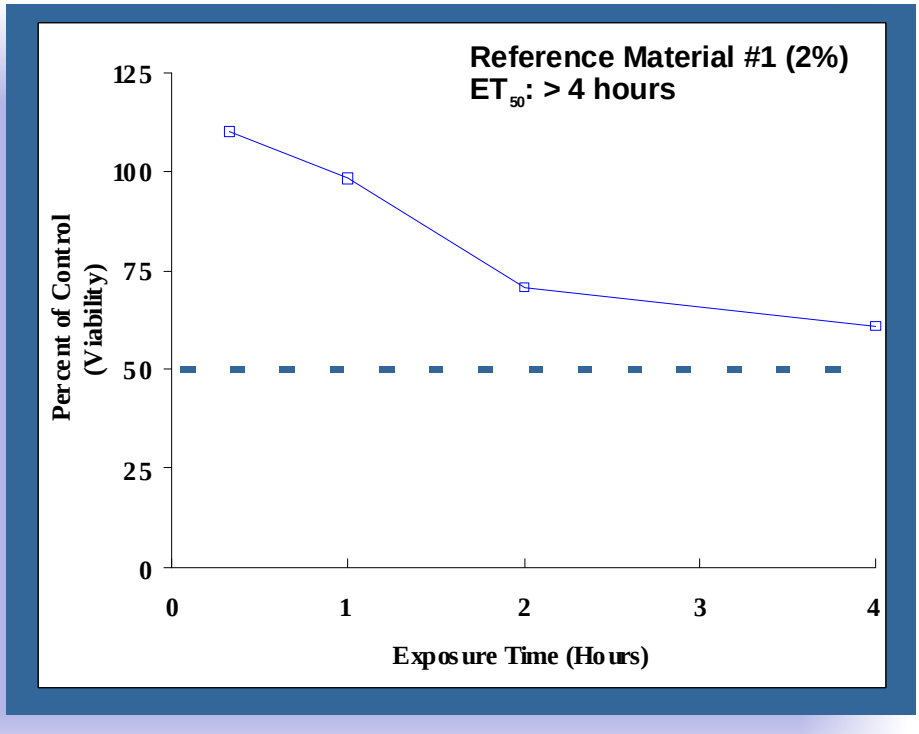


Figure 5. Reference material #2 (Product #50) (see **Table 2**, tested at 2% - black and 10% - blue) exposure time response.

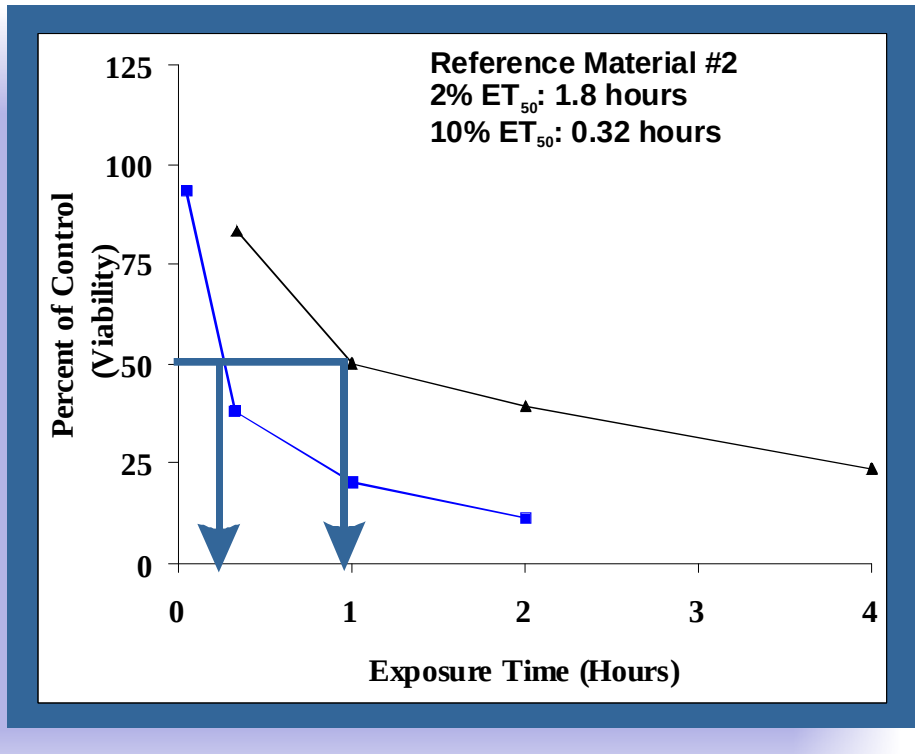
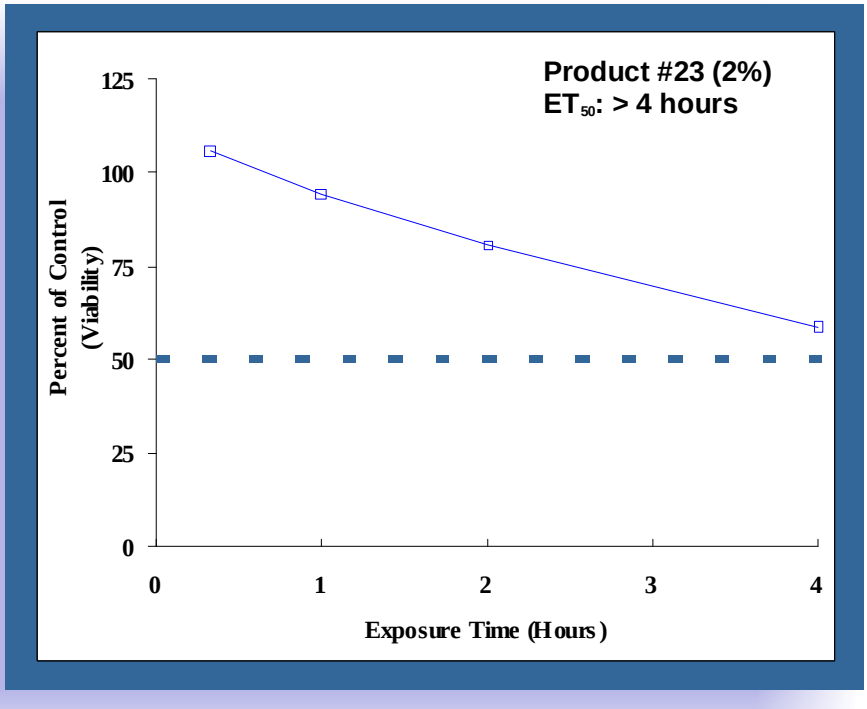
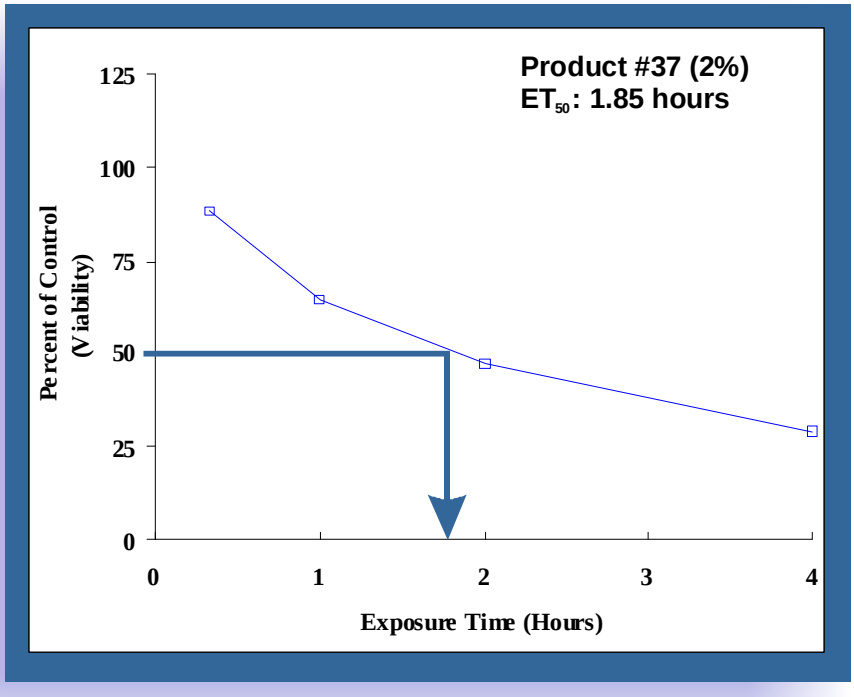


Figure 6. Products #23 (A) and #37 (B) (see **Table 2**, tested at 2%) exposure time response.

A.)



B.)



DISCUSSION

All products tested using the program outlined herein and based on the in vitro EpiOcular™ assay were predicted to fall in the mild to no irritation range based on the existing prediction model and by comparison with the Reference materials. As expected, the ET_{50} values obtained for the 10% dilutions of the test articles were shorter (showed greater cytotoxicity) as compared to the values obtained when 2% dilutions were used. The cytotoxicity results of the test materials can still be compared since the Reference materials were tested along with the test materials at 2% and 10% dilutions. The positive control ET_{50} results in EpiOcular™ tissue were reproducible and consistent with results from previous studies over the last decade. This is important because it implies the model will provide the stable platform needed when a long term in vitro testing program is being envisioned.

Although the data showed some variability within surfactant systems, we feel confident that this is a reflection of other ingredients within the formulation and not a reflection of a weakness in the model. In two instances, we used the results of these tests to inform the decision-making process to move forward with one formulation over another. The lack of adverse consumer experiences in these cases appears to support these decisions well.

REFERENCES

Berridge, M. V., Tan, A. S., McCoy, K. D., and Wang, R. (1996) The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts, Biochemica, 4, 14-19.

Blazka, M. E., Harbell, J. W., Klausner, M., Merrill, J., Kubilus J., Kloos, C., and Bagley, D. M. (2003) Evaluating the ocular irritation potential of 54 test articles using the EpiOcular™ human tissue construct model (OCL-200). Presented at the 2003 Society of Toxicology Meeting.

Kay, J. H., Calandra, J. C. (1962) Interpretation of eye irritation tests, J. Soc. Cosmetic Chem., 13, 281-289.

McCain, N. E., Binetti, R. R., Gettings, S. D., and Jones, B. C. (2002) Assessment of ocular irritation ranges of market-leading cosmetic and personal-care products using an in vitro tissue equivalent, The Toxicologist, 66, 243.

ACKNOWLEGEMENTS

The authors would like to thank Amanda Ulrey and Dr. Gertrude-Emilia Costin of IIVS for their assistance with poster preparation.

Institute for In Vitro Sciences, Inc.
30 W. Watkins Mill Road, Suite 100
Gaithersburg, MD 20878
Ph: 301.947.6523
Fx: 301.947.6538
For information, please contact: aulrey@iivs.org
or visit our website: www.iivs.org

Institute for In Vitro Sciences, Inc.

Advancing Science & Animal Welfare Together