The Development and Utilization of an In Vitro Safety Testing Program for Hair Conditioners

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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 has long been the goal of many international companies. This concern has become even more important with the requirement of the Seventh Amendment to the Cosmetics Directive that after 2009 animal testing cannot be used to assess the eye or skin irritation potential of either cosmetics formulations or ingredients. To address this problem, the Alberto-Culver Company has developed a program to support the ocular safety evaluation of certain hair conditioners. This 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 (ET_{50}; MTT activity reduced to 50% of the control condition). Twenty-eight different formulations primarily based on either single or dual quaternary ammonium compound (quats) systems utilizing various combinations of seven different quats have been evaluated in the model. Potential safety of the materials was assessed by comparison to a benchmark material having a well established safety profile in commerce. Twenty-seven of the materials, including the benchmark, had ET_{50} values of 24 hours or greater, indicating that they were quite mild. The effectiveness of the system has been assessed by comparing the in vitro results with consumer experience information.

INTRODUCTION

Quaternary ammonium compounds have great utility in hair conditioning products as these cationic materials are substantive to the hair shaft and provide tactile softening effects. Unfortunately, along with these desirable properties, these compounds by themselves may cause some irritation to the eye upon accidental exposure. In product development, possible synergistic effects need to be assessed when pairing multiple quaternary ammonium salts.

In order to ascertain that the use of this dual conditioning agent system did not produce a concurrent increase in the ocular irritation potential of the prototype product, we tested two mono quaternium ammonium agents and ten dual quaternium ammonium salt systems in final formulations, using the EpiOcular™ model. 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 (Stern, 1998; 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).

The EpiOcular™ model was used for in vitro safety assessment studies performed by The Institute for In Vitro Sciences (IIVS) in collaboration with the Alberto-Culver Company. In this particular study, 28 conditioners were tested and the results of the EpiOcular™ assay were then compared to existing consumer adverse experience data.

MATERIALS AND METHODS

Media and reagents

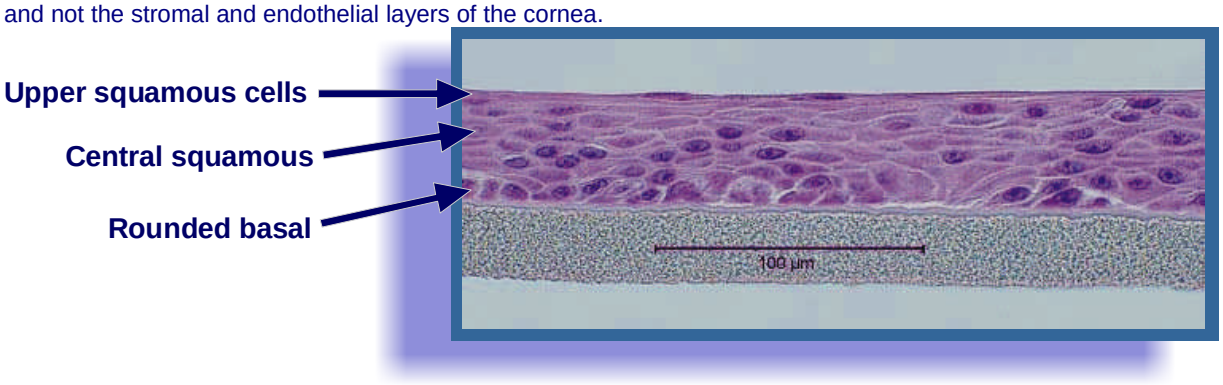
- Assay Medium and the EpiOcular™ Tissue (OCL-200) - MatTek Corporation (Ashland, MA).
- Dulbecco's Modified Eagle's Medium (DMEM) containing 2 mM L-glutamine (MTT Addition Medium), the phosphate-buffer saline (PBS), the Ca++ and Mg++-Free Dulbecco's Phosphate Buffered Saline ($Ca^{++}Mg^{++}$-Free DPBS) (pH 7.0±0.5), and the sterile, deionized water - Quality Biological (Gaithersburg, MD).
- 10X stock solution of 3-(4, 5 - dimethylthiazol-2-yl) - 2, 5 - diphenyltetrazolium bromide (MTT)
- (10 mg/ml MTT in PBS) and the extraction medium (isopropanol) - Sigma Aldrich (St. Louis, MO).

Test system

The EpiOcular™ Human Cell Construct model (OCL-200) is a three-dimensional non-keratinized tissue construct modeling the human corneal epithelium. The construct is composed of normal human derived epidermal keratinocytes (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).

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.



Test material administration

Test materials were liquid conditioners of a viscous or semi-viscous nature (Table 1). In consideration of relevant consumer exposure, test materials were tested without dilution (neat). Each dosed tissue was fully covered and exposed to the test materials for 4, 8, 16, and 24 hours.

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 per culture for 15 and 45 minutes, in duplicate cultures. The results of an assay were considered to be valid only when the positive control result fell within the established acceptable range generated from the IIVS positive control historical results (Table 2). The negative control (sterile, deionized water) was tested at multiple exposure times to provide an appropriate 100% viability response in the range of exposure times up to 24 hours. Thus, 100 µL of sterile, deionized water per culture were tested in duplicate cultures for 15 minutes, 4, 8 and 24 hours.

Reference material

As an added assessment tool, a material with similar chemistry and well established safety assessment data was selected and tested in parallel with other test materials as the reference material (Table 3 - Product #15).

Treatment of cultures

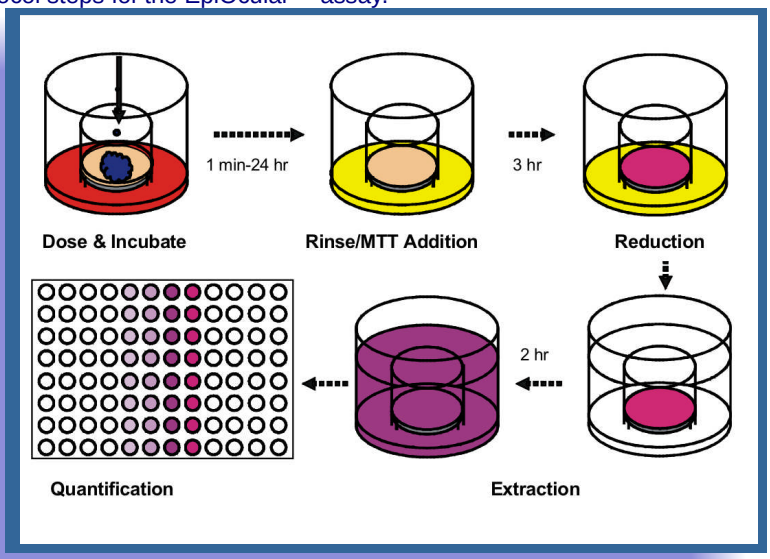
A schematic of the assay procedure is given in Figure 2. One hundred µL of viscous and semi-viscous liquids were applied directly on the tissue using a positive displacement pipet so as to cover the upper surface. Time points used to evaluate the irritation potential of each test material were 4, 8, 16, and 24 hours. These time points are typical for long time exposure for products in mild to ultra mild category. A general prediction model for this type of protocol using cosmetics and personal care products was proposed by Stern, 1998 and is shown in Figure 3. The long exposure times used in this study were selected based on previously determined cytotoxicity responses induced by this type of test material. Two tissues were used for each exposure time per test material.

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 or in the insert supporting membrane after the rinsing step. In such a situation the amount of MTT directly reduced by the test material would be evaluated as if the MTT signal was generated by viable cells. Thus the total MTT reduced would appear to be notably higher (implying that the test material was less cytotoxic) when in fact significant cytotoxicity may have been induced. To assess the ability of each test material to directly reduce MTT, a 1.0 mg/mL MTT solution was prepared and 100 µL of liquid test materials were added to 1 mL of the MTT solution. The mixture was then incubated in the dark at 37±1ºC in a humidified atmosphere of 5±1% CO2 in air (standard culture conditions) for approximately one hour. If the MTT solution color turned blue/purple, the test material was presumed to have reduced the MTT. Water insoluble test materials often show direct reduction (darkening) only at the interface between the test article and the medium.

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. Freeze-killed tissue was prepared by placing untreated EpiOcular™ constructs in a 20±5ºC freezer at least overnight, thawing to room temperature, and then refreezing until use. To test for residual test material reduction, killed tissues were treated with the test material in the normal fashion. Each test material was evaluated for at least the shortest and longest exposure times in single replicate killed tissues. All assay procedures were performed as for the viable tissue. At least one killed control treated with sterile deionized water (negative killed control) was tested for the

longest regular control exposure time since a small amount of MTT reduction is expected from the residual NADH and associated enzymes within the killed tissue. The test materials used in this study showed an insignificant effect in the killed control experiments.

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



MTT tissue viability assessment

Following exposure, the test material was removed by rinsing the cultures in Ca++ and Mg++ Free 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 (i.e. >24 hours).

RESULTS

Figures 4, 5 and 6 are examples of the exposure time response curves generated in a typical EpiOcular™ assay conducted for this program. For the reference material (Product #15 - Figure 4), no significant cytotoxicity was measured at any exposures up to the longest test material exposure time of 24 hours; therefore, an ET_{50} of > 24 hours was assigned. The Product #3 (Figure 5) caused a decrease in the level of MTT reduction as the exposure time increased. The ET_{50} was interpolated between the exposure times of 8 hours (mean viability of 86.0%) and 16 hours (mean viability of 45.9%). The positive control ET_{50} (36.9 minutes) was within the current acceptable range of 15.9 minutes to 39.1 minutes applicable to this study (Figure 6).

TABLE 1. Concentration of active ingredients in test materials.

Quat 1	Active level-1	Quat 2	Active level-2	Quat system
Stearalkonium chloride	B	NA	NA	Mono 1
Cocamidopropyl PG-dimonium chloride phosphate	A	NA	NA	Mono 2
Stearalkonium chloride	A	Cetrimonium chloride	A	Dual 1
Stearalkonium chloride	B	Cetrimonium chloride	A	Dual 2
Stearalkonium chloride	A	Cetrimonium bromide	A	Dual 3
Quaternium-18	A	Isostearamidopropyl ethyldimonium ethosulfate	A	Dual 4
Stearalkonium chloride	A	Cetrimonium chloride	A	Dual 5
Quaternium-18	A	Quaternium-80	A	Dual 6
Stearalkonium chloride	A	Cetrimonium chloride	A	Dual 7
Quaternium-18	B	Quaternium-80	A	Dual 8
Quaternium-18	B	Quaternium-80	A	Dual 9
Stearalkonium chloride	A	Cetrimonium chloride	B	Dual 10

A< 1%; 1%<B<5%

TABLE 2. Stability of positive control values.

Test Material	Range (min)	Mean ET_{50} value (min)	Standard Deviation (min)	CV
0.3% Triton -X-100 (Combined data from MatTek and IIVS)	15.3 - 36.9	26.1	5.4	20.7%
0.3% Triton -X-100 (IIVS only - through July 2007)	15.9 - 39.1	27.5	5.8	21.2%

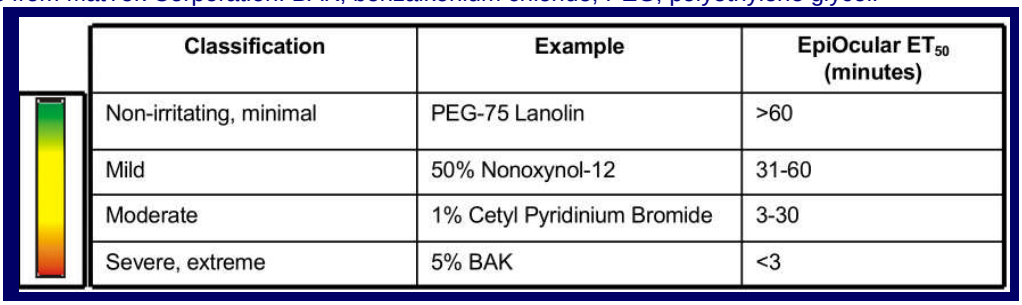
TABLE 3. Test results and consumer experience.

Product	Quaternary system	Consumer adverse experience	ET_{50} (hours)
1	Mono 1	0	>24
2	Mono 2	0	>24
3	Dual 1	0	15.2
4	Dual 2	0	>24
5	Dual 3	0	>24
6	Dual 4	0	>24
7	Dual 4	0	>24
8	Dual 5	0	>24
9	Dual 6	0	>24
10	Dual 6	0	>24
11	Dual 6	0	>24
12	Dual 6	0	>24
13	Dual 7	0	>24
14	Dual 8	+	>24
15 (*Reference material*)	Dual 8	0	>24
16	Dual 9	+	>24
17	Dual 10	0	>24
18	Dual 10	0	>24
19	Dual 10	0	>24
20	Dual 10	+	>24
21	Dual 10	0	>24
22	Dual 10	0	>24
23	Dual 10	0	>24
24	Dual 10	0	>24
25	Dual 10	0	>24
26	Dual 10	0	>24
27	Dual 10	0	>24
28	Dual 10	0	>24

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.

ET_{50}: when all of the test material exposure time points showed greater than 50% viability, the ET_{50} value was presented as greater than the longest test material exposure time (i.e., "> 24 hours").

FIGURE 3. A prediction model for personal care and cosmetic products based on results from Stern, 1998 with examples from MatTek Corporation. BAK, benzalkonium chloride; PEG, polyethylene glycol.



Classification	Example	EpiOcular ET_{50} (minutes)
Non-irritating, minimal	PEG-75 Lanolin	>60
Mild	50% Nonoxynol-12	31-60
Moderate	1% Cetyl Pyridinium Bromide	3-30
Severe, extreme	5% BAK	<3

DISCUSSION

All conditioner systems tested with the in vitro EpiOcular™ protocol in this program were predicted to have an acceptable mildness to the eye based on the existing prediction model and by comparison with the reference material. One dual quaternium ammonium salt system proved to be somewhat less tolerated (ET_{50} = 15.2 hr) (see **Table 3** and **Figure 5**), although this is still considered to be very mild according to the current prediction model. One possible explanation is that this lower ET_{50} could be attributable to other ingredients in the formulation that were not common to the remaining formulations. The results of these in vitro tests were compared to subsequent consumer experience. Only three products showed any evidence of consumer experience with irritation, none of which correlated with the lower ET_{50} values. However, when normalized to sales data (see **Table 3** for description of adverse effects rating system) these idiosyncratic experiences were considered insignificant with respect to the volume of product sold.

The lack of irritation seen during in vitro testing was determined to be in high concordance with consumer experience. 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.

FIGURE 4. Reference material (see Table 3, Product #15) exposure time response.

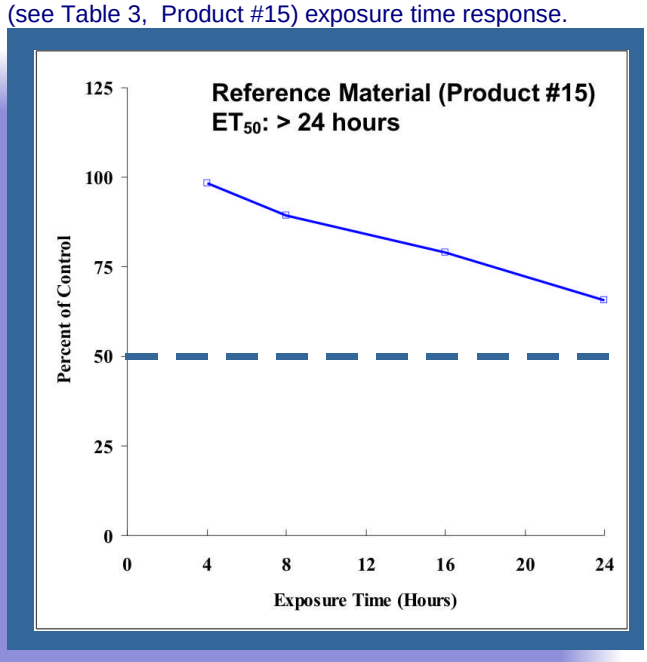


FIGURE 5. Product #3 (see Table 3) exposure time response.

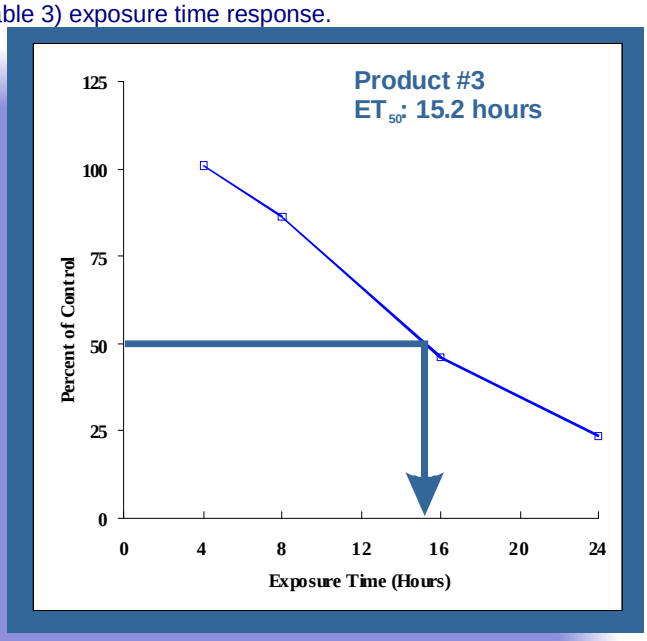
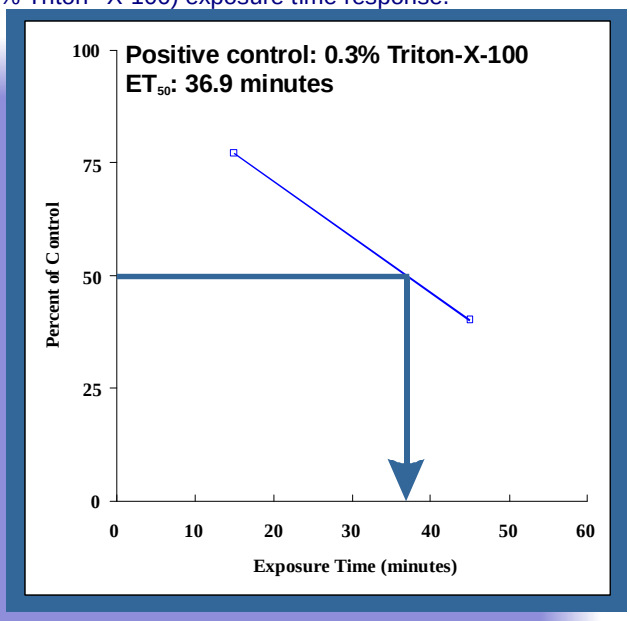


FIGURE 6. Positive control (0.3% Triton®-X-100) exposure time response.



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ACKNOWLEGEMENTS

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

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