Modification in Procedure of the Reconstructed Human EpiDermis (RhE) Phototoxicity Assay

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INTRODUCTION

Phototoxicity, or photoirritation, is a toxic response elicited by the exposure of skin to certain chemicals and subsequent exposure to light. The reconstructed human epidermis (RhE) model has been used in the evaluation of photosafety for many decades and was recently adopted as part of Test Guideline 498: In vitro phototoxicity: Reconstructed human epidermis phototoxicity test method by the Organisation for Economic Cooperation and Development (OECD). The RhE model is composed of normal human-derived epidermal keratinocytes stratified to form multiple layers, which include viable cell layers and a functional *stratum corneum*, which mimics the outermost layer of human skin and provides a barrier function. The RhE tissue model allows for the application of a wide variety of test materials of differing physicochemical properties, including ingredients and complex formulations. The typical exposure is a topical application of test material, and this testing platform is also amenable to a basal exposure (i.e., application of test material directly into the culture medium to mimic a systemic exposure). Further, this test platform is able to overcome solubility limitations of other *in vitro* models (e.g., OECD TG 432: *In Vitro* 3T3 NRU Phototoxicity Test).

The purpose of this investigation was to evaluate the phototoxicity potential of the proficiency substances described in OECD TG 498 using the EpiDerm™ Skin Model (MatTek Corporation, MA, USA) and two different procedural rinsing approaches. Each proficiency chemical was evaluated at three concentrations in the presence and absence of UVA/visible light. The concentrations evaluated and the solvents used to prepare each proficiency chemical are presented in **Table 1** and **Table 2**. The Dermalight SOL3 (UVATEC, Inc., USA) solar simulator equipped with the H1 filter, which allows UVA and visible light (i.e., ≥320 nm) and attenuating UVB, was used to expose the designated tissues to 6 J/cm^2 of UVA. The test material was removed from the tissues prior to the irradiation (designated as "pre-rinse") or removed from the tissues after irradiation (designated as "post-rinse"). The "post-rinse" aligned with the procedures described in the test guideline, where the substances are removed from the tissues after the UV light exposure. According to the TG 498, a "pre-rinse" method may be prescribed when a test materials' characteristics (e.g., dark colored or opaque material) could interfere with the light exposure. The "pre-rinse" method has been routinely performed at the Institute for In Vitro Sciences, Inc. (IIVS) for over a decade, largely due to the types of products evaluated, which consisted mostly of finished formulations that may have been dark colored (e.g., lipsticks) or opaque (e.g., creams). The assay controls were evaluated similarly (i.e., "pre-rinse" or "post-rinse" method) with appropriate responses.

Three phototoxic substances (chlorpromazine, anthracene, and bergamot oil) and three non-phototoxic substances (sodium dodecyl sulfate (SDS), 4-aminobenzoic acid (PABA), and octyl salicylate) (all sourced from Sigma-Aldrich, USA) were evaluated in at least one definitive assay. PABA was evaluated in additional trials at 3% (w/v) since this concentration resulted in an initial prediction of phototoxicity potential using the "pre-rinse" method only. All trial results of PABA at 3% are presented in **Figure 2**. A test material was considered to have phototoxicity potential if viability of the tissues exposed in the presence of UVA showed a difference of ≥30% as compared to the viability of the tissues exposed in the absence of UVA at any tested concentration.

EXPERIMENTAL DESIGN

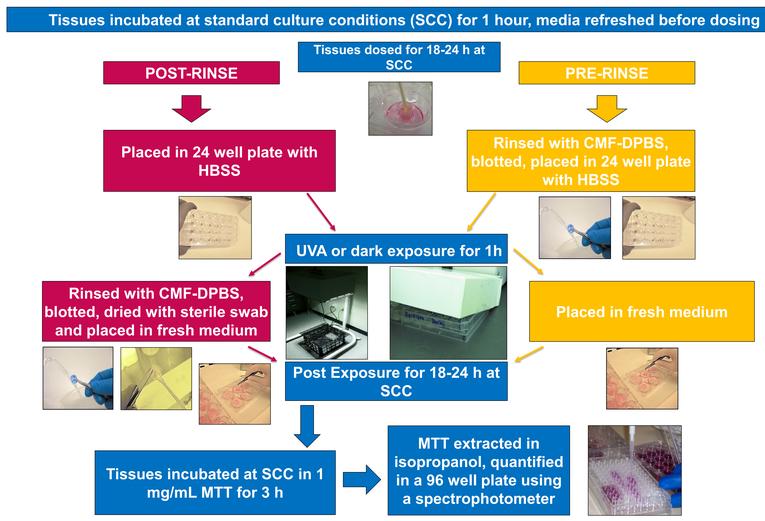


Figure 1. Summary Flowchart of Assay Steps Showing Differences in Test Material Removal Methods

Each proficiency material was evaluated at three concentrations (see **Table 1**) in a solvent suggested in OECD TG 498 and confirmed by solubility assessment at IIVS (See **Table 2**). For anthracene, which was prepared in Hanks' Balanced Salt Solution (HBSS) containing 1% ethanol, the anthracene was initially prepared at 100X in ethanol and diluted 1:100 into HBSS to create the final tested 1X concentrations. All other materials were prepared directly in solvent. The volume of the dose applied to the tissues aligned with OECD TG 498 (i.e., 50 µL, or 25 µL for oil soluble materials). Each concentration was topically applied to six tissues: two tissues designated for exposure in the absence of light and four tissues designated for exposure in the presence of light. Of the four tissues exposed in the presence of light, two tissues were designated as "pre-rinse" and two tissues were designated as "post-rinse". The treatments were removed by rinsing the tissues with sterile calcium & magnesium free Dulbecco's Phosphate Buffered Saline (CMF-DPBS) prior to irradiation (i.e., "pre-rinse") or after irradiation (i.e., "post-rinse"). Sterile cotton swabs soaked in buffered saline were used in an attempt to remove the test material from the tissues when residual test material was noted on the tissues after the rinsing process (i.e., twenty rinses delivered to the tissues using a squirt bottle). All tissues were placed into 24-well plates containing Hanks' Balanced Salt Solution (HBSS) for the UVA or dark exposure at room temperature. After the 60 minute incubation at room temperature, the tissues exposed in the dark condition were rinsed (i.e., in the same timeframe as the "post-rinse" tissues). For the "post-rinse" method, the tissues were rinsed with sterile CMF-DPBS and gently dried with sterile cotton swabs to remove any excess moisture. After the irradiation or dark exposure and treatment removals, the tissues received an overnight incubation period at standard culture conditions (SCC) (i.e., 5%±1% CO_2 and 37°±1°C in humidified air) prior to the assessment of tissue viability using the MTT assay.

DATA ANALYSIS & PREDICTION MODEL

$$\% \text{ Viability} = \frac{\text{corrected OD}_{550} \text{ of UVA/Dark Exposure Matched Treatment Group}}{\text{corrected OD}_{550} \text{ of UVA/Dark Exposure Matched Solvent Control}} \times 100$$

The viability of the tissues that were not irradiated (i.e., dark exposure, -UVA) were calculated relative to the solvent controls that were also not irradiated. Similarly, the viability of tissues that were irradiated (i.e., +UVA) were calculated relative to the solvent controls that were also irradiated. Solvent controls were run concurrently with the treatment groups. The mean viability results for each test material at each concentration +/- 1 standard deviation are presented in **Figures 2-8**.

If any concentration induced ≥30% decrease in viability in the presence of UVA compared to the viability in the absence of UVA, then the test material was considered to have exhibited a phototoxic response. The % decreases in the viability of each treatment group in the presence of UVA as compared to the absence of UVA are presented in **Table 1**.

RESULTS

Proficiency Substance	Conc. (w/v)	% Difference in Viabilities		Phototoxic in Post-Rinse?*	Phototoxic in Pre-Rinse?*
		Post Rinse Method	Pre Rinse Method		
Chlorpromazine	0.06%	1.5	1.2	Yes	Yes
	0.02%	45.6	56.6		
	0.006%	13.3	4.8		
Anthracene	0.1%	83.8	84.8	Yes	Yes
	0.03%	97.2	96.6		
	0.01%	88.3	86.6		
Sodium Dodecyl Sulfate (SDS)	0.3%	-0.1	0.4	No	No
	0.1%	-2.7	-1.0		
	0.03%	3.7	-5.9		
4-Aminobenzoic Acid (PABA)	10%	13.9	14.9	No	No
	3%	0.1	14.7		
	1%	1.3	6.0		
Octyl Salicylate	10%	-9.4	-0.2	No	No
	3%	-1.2	3.2		
	1%	-9.2	20.1		
Bergamot Oil	10%	67.7	60.4	Yes	Yes
	3%	83.2	78.0		
	1%	78.9	33.0		

Table 1. Summary % Difference in Viabilities and Phototoxicity Prediction

Percent difference in viability of tissues exposed in the presence of UVA to the viability of the tissues exposed in the absence of UVA at the "pre-rinse" and "post-rinse" conditions. The phototoxicity prediction is based on the model presented in OECD TG 498 (≥ 30% difference indicates potential phototoxicity).

***According to OECD TG 498, a difference of ≥ 30% is predictive of phototoxic potential, and <30% difference is predicted as non phototoxic.**

Solvents Used to Prepare Proficiency Chemicals					
Chlorpromazine (CAS# 69-09-0)	Anthracene (CAS# 120-12-7)	Sodium Dodecyl Sulfate (SDS) (CAS# 151-21-3)	4-Aminobenzoic Acid (PABA) (CAS# 150-13-0)	Octyl Salicylate (CAS# 118-60-5)	Bergamot Oil (CAS# 8007-75-8)
HBSS	HBSS w/ ethanol intermediate	HBSS	Mineral Oil	Mineral Oil	Mineral Oil

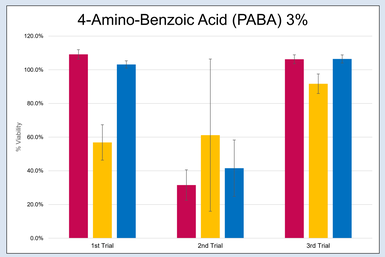
Table 2. Proficiency Substances and Solvents Used

Post-Rinse + UVA = Pre-Rinse + UVA = Without UVA/Dark =

Solubility and Variable Response of PABA at 3%

PABA at 3% was evaluated in three trials. In the first trial, the results indicated phototoxicity in the "pre-rinse" tissues only (46.3% difference in viability). In the second trial, all of the viabilities were inconsistent with the previous results, showing increased levels of cytotoxicity, and further, the replicate tissue responses were highly variable. The third trial resulted in similar viabilities for all treatment groups and aligned with the expected responses as per the OECD TG.

In all study trials, the PABA 3% dilution was not completely soluble. The 3% dilution was described as a suspension with white particles in all study trials. While solubility is not a limiting factor of this test method, dilutions that are not completely soluble in any test method can impact the actual concentration tested and may contribute to variability in responses.



Reference: OECD Test Guideline No. 498 In vitro Phototoxicity: Reconstructed Human Epidermis Phototoxicity test method, 14 June 2021

Reference: INVITTOX Protocol 121. EpiDerm™ Phototoxicity Assay. ECVAM DB-ALM; 1999. *http://ecvam-dbalm.jrc.ec.europa.eu/*

RESULTS

Post-Rinse + UVA = Pre-Rinse + UVA = Without UVA/Dark =

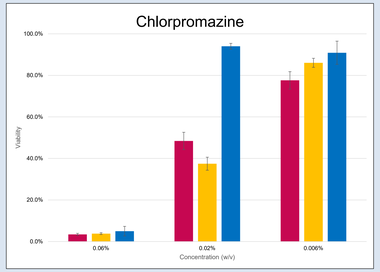


Figure 3. Viability of Chlorpromazine at 0.06%, 0.02% and 0.006% in HBSS

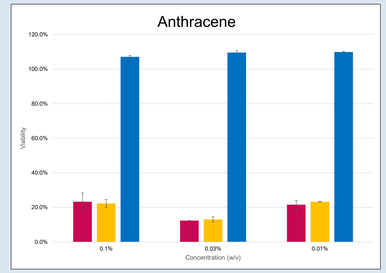


Figure 4. Viability of Anthracene at 0.10%, 0.03% and 0.01% in HBSS (with EtOH intermediate)

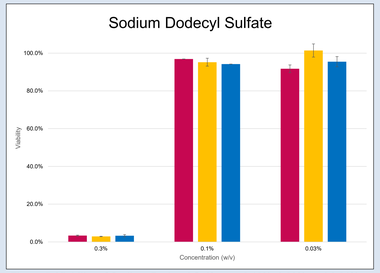


Figure 5. Viability of Sodium Dodecyl Sulfate at 0.10%, 0.03% and 0.01% in HBSS

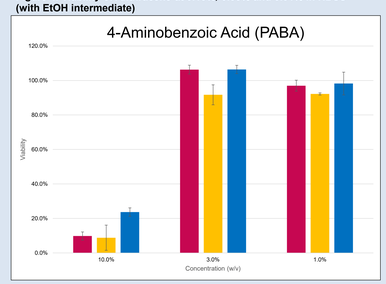


Figure 6. Viability of 4-Aminobenzoic Acid at 10%, 3% and 1% in Mineral Oil

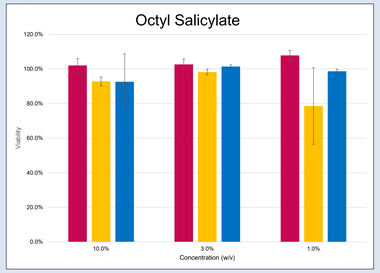


Figure 7. Viability of Octyl Salicylate at 10%, 3% and 1% in Mineral Oil

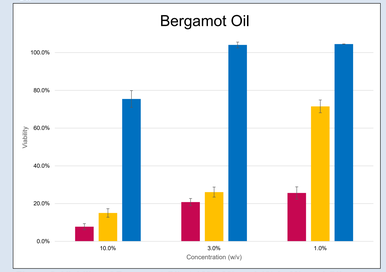


Figure 8. Viability of Bergamot Oil at 10%, 3% and 1% in Mineral Oil

CONCLUSIONS & CONSIDERATIONS

All proficiency materials aligned with the expected result for phototoxicity potential using either rinsing approach. This supports that a "pre-rinse" method could be incorporated into testing protocols performed under the guidance of OECD TG 498.

The "pre-rinse" method may be advised, especially for materials that are opaque or insoluble. Test materials that partially or completely block out UVA light may result in an inadequate exposure to UVA and thus affect the ability of this assay to predict phototoxicity potential. Previous research at IIVS has suggested that opaque materials (i.e., creams) spiked with a phototoxic material (e.g., chlorpromazine) may not be adequately assessed for phototoxicity potential when using the post-rinse methodology, especially if the cream (solvent) can act as a UV filter (data not shown).

The 18-24 hour treatment exposure time outlined in TG 498 provides sufficient time for penetration into the living tissue prior to the irradiation exposure. Therefore, removal of the treatment prior (i.e., "pre-rinse" method) should not impact the ability to adequately assess phototoxic materials that are able to penetrate the skin. Test materials that fail to penetrate the skin barrier (i.e., stratum corneum) may pose limited phototoxicity risk unless the material becomes activated during irradiation at the surface of the skin with increased penetration across the skin. Further, for clinical photosafety testing, residual test material not absorbed into the skin may be removed prior to irradiation.

While the OECD TG 498 test method may overcome the solubility limitation discussed in the monolayer Balb/c 3T3 Phototoxicity test (OECD TG 432), solvent selection should also be carefully considered for the RhE multi-concentration method. As demonstrated with repeated trials of PABA at 3%, solubility may be a contributing factor to the variability. Further testing of PABA with a different solvent (e.g., sesame oil or ethanol) could provide resolution.

The initial work showed a possibility of increased assay sensitivity when using the "pre-rinse" method for PABA at 3%. The 3% PABA produced a >30% difference in the "pre-rinse" method, but not the "post-rinse" method (-6.0% difference). This was not reproduced in two additional trials. Additional testing of a larger subset of test materials could further evaluate the assay sensitivity using the "pre-rinse" and "post-rinse" method.