CHOOSING THE APPROPRIATE SOLVENT FOR SOLID MATERIALS TESTED IN THE BOVINE CORNEAL OPACITY AND PERMEABILITY (BCOP) *IN VITRO* ASSAY

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ABSTRACT

In compliance with OECD Test Guideline 437 for eye irritation (BCOP assay), non-surfactant solid materials are typically tested as 20% dilutions prepared in 0.9% sodium chloride solution, distilled water, or other solvent that has been demonstrated to have no adverse effects on the test system. However, the limited solubility of some chemicals adds technical challenges in finding a vehicle that would ensure the material's availability to the excised corneas and that itself would not affect the test system. In this study, we evaluated five solvents frequently used in the BCOP assay: distilled water, mineral oil, corn oil, polyethylene glycol (PEG)-400, and methocel solution (0.5%). Based on the available classification systems, our preliminary data showed that water, methocel, mineral oil and corn oil were predicted as non-irritants, while PEG-400 was predicted as a mild irritant. To demonstrate the influence of the type of solvent on the outcome/prediction of the BCOP assay for solid materials, we tested a 20% suspension of benzoic acid (BA) prepared in these solvents. BA has a non-polar benzoic ring that would preferably dissolve in non-polar solvents and a polar acidic group with affinity for polar solvents, thus making it a good model for testing its effect on corneas when dissolved in various solvents. Previous animal tests reported moderate to severe eye irritation induced by BA. Our results demonstrated that when mixed in water, mineral oil, corn oil, or methocel, BA was predicted to be a corrosive/severe irritant, while it was predicted to be a moderate irritant when mixed in PEG-400. These results support the need for further investigation of the solvent's influence in the BCOP assay to allow the correct prediction of the irritation potential of solid materials.

INTRODUCTION

To investigate the influence of vehicle in the *in vitro* eye irritation Bovine Corneal Opacity and Permeability (BCOP) assay, we assessed and compared the eye irritation of five solvents: sterile deionized water (also the assay's negative control), mineral oil, corn oil, PEG-400 and 0.5% methocel (Methylcellulose) solution. These solvents are routinely utilized for the Draize rabbit eye test when the test substance is not directly applicable. These solvents were subsequently used to prepare the 20% dilution of benzoic acid (BA) tested per OECD TG 437 (1). Benzoic acid is used in topical drugs such as anti-fungal creams or in cosmetics, thus having the potential to induce irritation through accidental exposure to the eye. Previous animal tests reported moderate to severe eye irritation induced by BA (2-6). Benzoic acid has a non-polar benzene ring that would preferably dissolve in non-polar solvents and a polar acidic group with affinity for polar solvents, thus making it a good model for testing its effects when dissolved in various solvents.

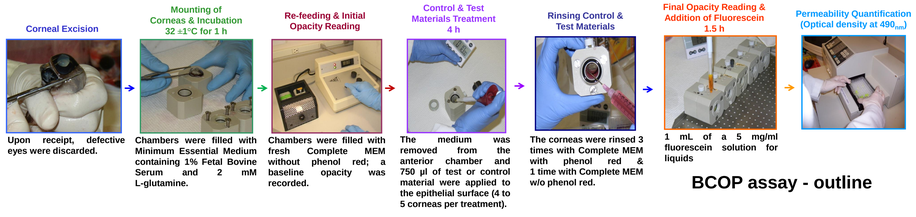
MATERIALS & METHODS

- *In Vitro* Score = Mean Opacity Value + (15 x Mean OD_{490} Value)
- Histology analysis: Hematoxylin & Eosin (HE) staining
- Prediction models (7-8):

In Vitro Score:

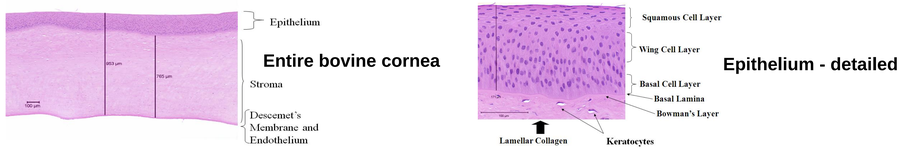
0 to 3 = non irritant — 3.1 to 25 = mild irritant

25.1 to 55 = moderate irritant — 55.1 and above = corrosive/severe irritant



BCOP assay - outline

Structural architecture of bovine cornea



Entire bovine cornea — **Epithelium - detailed**

SOLVENTS

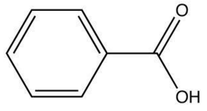
Table 1. BCOP *in vitro* scores - solvents

Solvent	Mean Opacity	Mean OD490 (Permeability)	*In vitro* score	Predicted irritation
Mineral oil	-2.0	-0.001	-2.0	Non-irritant
Corn oil	-1.0	0.002	-1.0	Non-irritant
PEG-400	4.0	0.051	4.8	Mild irritant
0.5% methocel	-2.7	-0.002	-2.7	Non-irritant

BENZOIC ACID DILUTED IN SOLVENTS

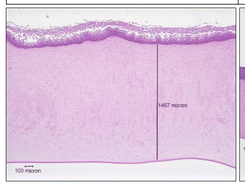
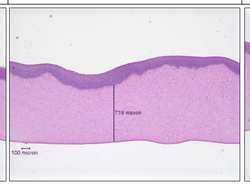
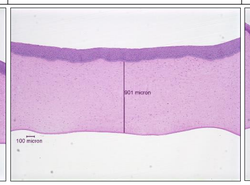
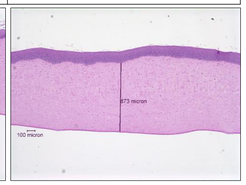
Table 2. BCOP *in vitro* scores – 20% (w/v) BA diluted in solvents

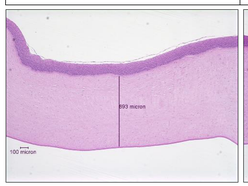
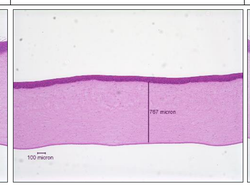
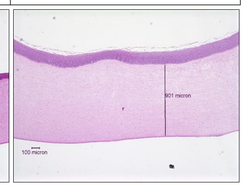
Test material	Mean Opacity	Mean OD490 (Permeability)	*In vitro* score	Predicted irritation
Benzoic Acid in Deionized water	112.0	0.004	112.1	Severe irritant
Benzoic Acid in Mineral oil	202.7	0.005	202.7	Severe irritant
Benzoic Acid in Corn oil	158.7	-0.001	158.7	Severe irritant
Benzoic Acid in PEG-400	42.7	0.298	47.1	Moderate irritant
Benzoic Acid in 0.5% methocel	85.7	0.003	85.7	Severe irritant
Imidazole (positive control)	73.7	1.142	90.8	Severe irritant



Benzoic acid

RESULTS

	Positive control (Imidazole)	Deionized water	Mineral oil	Corn oil	PEG-400	0.5% methocel
						
Epithelium	• Nonviable • Cytoplasmic contents were coagulated in small clusters and highly eosinophilic • Nuclei showed marked abnormal chromatin condensation	• Basal cell layer well-formed • Columnar-cell region directly attached to the Bowman's layer	Similar to water-treated corneas	Similar to water-treated corneas	• Sloughing of the squamous layer along with nuclear vacuolation • Wing cell layer had cytoplasmic and nuclear vacuolation along with loss of cell to cell adhesion	Similar to water-treated corneas
Stroma	• Swelling in the upper through deeper stroma • Keratocytes showed a marked increase in the frequency of cells with nuclear pyknosis or other forms of abnormal chromatin condensation	• Stromal elements well-organized • Collagen matrix fibers with dispersed keratocytes			Similar to water-treated corneas	
Endothelium	• Lost	• Descemet's membrane prominent				

	20% (w/v) BA in Deionized water	20% BA (w/v) in Mineral oil	20% BA (w/v) in Corn oil	20% BA (w/v) in PEG-400	20% BA (w/v) in 0.5% methocel
					
Epithelium	• Sloughing of surface squamous layer • Abnormal chromatin condensation in the nuclei throughout the squamous, wing and basal cell layers		• Flattened and condensed • Loss of structure in the wing and basal cell layers		• Sloughing of the squamous layer along with cytoplasmic and nuclear vacuolation • Abnormal condensation in the entire epithelium through the squamous, wing, and basal cell layers
Stroma	• Stroma directly beneath Bowman's layer had minimal collagen swelling with a few enlarged keratocyte nuclei with abnormal chromatin condensation	• Stroma thicker than the water-treated corneas • Stroma directly beneath Bowman's layer had moderate collagen swelling with enlarged keratocyte nuclei containing abnormal chromatin condensation • Lower stroma similar to the water-treated corneas	Stroma appeared less swollen than the water-treated corneas		• Stroma thicker than the water-treated corneas • Stroma directly beneath Bowman's layer had minimal collagen swelling with vacuolated keratocyte nuclei and also keratocyte nuclei containing abnormal chromatin condensation • Lower stroma similar to the water-treated corneas
Endothelium	Similar to negative control-treated corneas				

CONCLUSIONS

SOLVENTS

1. The results showed that of the solvents used in the study, deionized water, mineral oil, corn oil and 0.5% methocel were predicted to be non-irritant. PEG-400 was predicted to be a mild irritant. The histology analysis confirmed the BCOP data by showing sloughing of the squamous layer of corneas treated with PEG-400.
2. When used to prepare the BA dilutions, all solvents induced high mean opacity scores and very low permeability scores, with the exception of PEG-400 which in reverse induced the lowest opacity score and the highest permeability score of all solvents used in the study.

BENZOIC ACID

3. BA was predicted by the BCOP assay as severe irritant when dissolved in deionized water, mineral oil, corn oil or 0.5% methocel and induced damage up to the mid depth stroma. When dissolved in PEG-400, BA was predicted to be a moderate irritant and limited its damaging effects to the epithelium. Furthermore, the stroma appeared better organized in corneas treated with BA dissolved in PEG-400 than in the negative control-treated corneas.

FUTURE CONSIDERATIONS

4. Our study emphasizes the importance of choosing the appropriate vehicle for solid materials when tested in the BCOP assay. The solvent may need to be selected based not only on previous animal data or on chemical properties but also on the form/dilution of the test material that the end-user comes in contact with in order to better reflect the effects upon human exposure.
5. Our results support the need for thorough investigation on the solvent's choice and influence in the BCOP assay to allow the correct prediction of the irritation potential of solid materials, particularly when validating the *in vitro* results against animal data.

REFERENCES

1. OECD. (2009). OECD GUIDELINE FOR THE TESTING OF CHEMICALS (OECD 437). Bovine Corneal Opacity and Permeability Test Method for Identifying Ocular Corrosives and Severe Irritants.
2. Bayer, A. G. (1978). Untersuchungen zur Haut- und Schleimhautverträglichkeit, Bayer AG Wuppertal.
3. Initial submission: primary eye irritation of benzoic acid to rabbits with cover letter dated 082892. Produced by: Monsanto Co. Date produced: Jan. 17, 1983. EPA/OTS 88-920008107. NTIS/OTS0546102.
4. Suberg, H. (1986). Benzoesäure DAB 8, Prüfung auf primär reizende/ätzende Wirkung am Kaninchenauge, Bayer AG data.
5. Beratergremium für umweltrelevante Altstoffe (BUA) der Gesellschaft Deutscher Chemiker (ed) BUA-Stoffbericht 145, December 1993.
6. RCC Notox, Primary skin irritation/corrosion study of benzoic acid in the rabbit (unpublished report). RCC Notox B.V., DD 's-Hertogenbosch, NL.
7. Gautheron, P., Dukic, M., Alix, D., and Sina, J. F. (1992). Bovine corneal opacity and permeability test: an in vitro assay of ocular irritancy. *Fundam. Appl. Toxicol* 18, 442-449.
8. Vanparys, P., Deknudt, G., Sysmans, M., Teuns, G., Coussement, W., and Van Cauteren, H. (1993). Evaluation of the bovine corneal opacity-permeability assay as an in vitro alternative to the Draize eye irritation test. *Toxicol. In Vitro* 7, 471-476.

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