Inter-laboratory Study of the Reproducibility of the Bovine Corneal Opacity and Permeability Assay: Investigations of Solid Test Substances

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ABSTRACT

The Bovine Corneal Opacity and Permeability Assay (BCOP) is an *ex vivo* assay, which may be used to assess the eye irritation potential of new chemicals and finished products. The BCOP assay has been accepted by several regulatory agencies for the identification of severe and corrosive ocular irritants, replacing the rabbit eye test. According to OECD Test Guideline 437 adopted in September 2009, two treatment protocols may be used; one for liquids and one for solids. Solids are tested as 20% (w/v) solutions or suspensions in deionized water. Freshly excised bovine corneas are mounted in special corneal holders and are treated with the 20% (w/v) test material dilutions for four hours at approximately 32°C. Changes in corneal opacity are measured using an opacitometer, and impairment of the corneal barrier function is determined by measuring fluorescein passage through the corneas. Histological evaluation of the treated corneas may be used to determine the degree and depth of injury at the tissue level. In this study, the reference standard solids recommended in the OECD TG 437 were tested in an inter-laboratory study. Overall, the results from the evaluation of solids were highly congruent between the two laboratories and to the historical data and for several substances histological evaluation improved the understanding of eye irritation effect. However, for chlorhexidine and dibenzoyl-L-tartaric acid there were inter-laboratory differences, which were further evaluated. For chlorhexidine, differences in results were attributed to different sources of the chemical. This study demonstrates the reproducibility of the BCOP assay when evaluating solid test substances. In parallel, the study compared the opacity scores from a newly developed opacitometer (BASF-OP2.0) to those of the standard device (OP-KIT). The comparison between the BASF-OP2.0 showed very little variability and overall corresponded very well with the OP-KIT values.

STUDY DESIGN AND PROCEDURES

- Solid test substances selected: 4 OECD 437 reference standards (ammonium nitrate, chlorhexidine, dibenzoyl-L-tartaric acid, imidazole), 5 from BCOP BRD (1-napthalene acetic acid, 4-carboxybenzaldehyde, aluminum hydroxide, EDTA, di-potassium salt, sodium oxalate)
- BCOP test was performed according to OECD TG 437 in BASF's (Ludwigshafen, Germany) and IIVS' (Gaithersburg, Maryland) laboratories:

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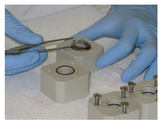
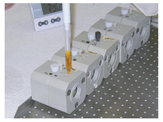
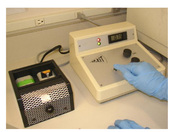
Corneal Excision	Mounting	Initial Opacity	Test Article Exposure	Rinsing	Fluorescein Addition	Permeability Endpoint
						
Upon receipt, eyes were examined and corneas free of defects were excised.	Corneas were mounted into chambers, and incubated for 1 hr. at 32 ± 1°C in cMEM .	cMEM was removed and refilled and the initial opacity was read on the OP-KIT and BASF-OP2.0 opacitometers.	750 µL of test substance (20% w/v) was applied to the anterior chamber or directly onto the epithelial surface for 4 hours at 32 ± 1°C.	Corneas were rinsed thoroughly to remove test substance, then chambers were refilled and final opacity taken.	1 mL of a 5 mg/mL fluorescein solution was added to the epithelial side of the corneas, and incubated at 32 ± 1°C for 1 hour.	Media was sampled from the posterior chamber and the optical density at 490 nm was quantified using a microplate reader.

- Sterile deionized water served as the negative control and imidazole diluted to 20% (w/v) served as the positive control
- Preparation of the corneas was carried out at both laboratories within 3 to 7 hours of harvest
- Opacity values were calculated from initial and final opacity values obtained from MC2 OP-KIT and BASF2.0 opacitometers at both labs



(a)

(b)

Figure 1. MC2 OP-KIT (a) and BASF-OP2.0 (b) Opacitometers. The BASF OP-2.0 was developed to enhance sensitive and reliable opacity measurement. Opacity values were obtained from both opacitometers at each lab for all corneas in this study. The performance of the BASF OP-2.0 was compared with that of the OP-KIT.

- Opacity and permeability values were used to calculate the In Vitro Irritancy Score (IVIS); also referred to as In Vitro Score
- In accordance with TG 437, substances with an IVIS> 55 were regarded as severe and labeled GHS Cat. 1., all others would be "not Cat. 1"
- According to Sina *et al.*, those with an IVIS of 25-55 were identified as moderate, and 0-25 were identified as mild eye irritants
- Histology was performed on select corneas to further assess degree and depth of injury
- Inter-laboratory results were compared for reproducibility and predictivity as compared to the results obtained from the *in vivo* Draize test

RESULTS

Table 1. Results of the *in vivo* and BCOP (opacity and permeability measurements) eye irritation tests. *In vivo* test performed at BASF, BCOP assay performed at IIVS and BASF.

Test Substance	In vivo (GHS)	IIVS Opacity Mean	IIVS Opacity SD	BASF Opacity Mean	BASF Opacity SD	IIVS Perm. Mean	IIVS Perm. SD	BASF Perm. Mean	BASF Perm. SD
1-Napthalene acetic acid	Cat. 1	56.3	19.7	160.3	47.3	0.034	0.027	0.015	0.025
4-Carboxybenzaldehyde	Cat. 2A	87.2	10.9	224.5	52.7	0.009	0.008	0.008	0.023
Aluminum hydroxide	n req	-0.3	0.14	23.4	16.3	0.002	0.003	0.011	0.024
Ammonium nitrate	2A/2B	5.7	0.9	5.8	2.8	0.015	0.009	0.020	0.023
Chlorhexidine	Cat. 1	57.5	4.93	122.7	19.6	0.015	0.003	0.024	0.014
Dibenzoyl-L-tartaric acid	Cat. 1	14.4	2.17	77.9	32.9	0.218	0.052	-0.001	0.000
EDTA, di-potassium salt	n req	-1.7	2.4	-4.8	3.7	0.007	0.007	0.035	0.062
Imidazole	Cat. 1	64.4	1.7	69.5	13.7	2.316	0.615	2.273	0.824
Sodium oxalate	Cat. 1	8.9	4.7	18.2	8.3	0.027	0.034	0.055	0.041

Table 2. Trials of Aluminum hydroxide, Chlorhexidine, and Dibenzoyl-L-tartaric acid. Testing Site, assay date, lot no., open or closed chamber dosing/rinsing, measures taken to solubilize, and In Vitro Score.

Test Substance	Testing Site	Date	Lot # (Supplier)	Open/Closed Chamber	Heat/ Sonicate	Data (In Vitro Score)
Aluminum hydroxide	IIVS	26-Aug-2010	MKBB5901 (Sigma)	open	No	-0.3
		28-Feb-2012				5.7
				closed		17.2
Chlorhexidine		24-May-2010	09312PH (Sigma)	open	No	57.7
		22 Dec-2010				57.6
			1312398V (BASF)	closed		97.8
				open		87.7
Dibenzoyl-L-tartaric acid		24-May-2010	00903MO (Sigma)	open	No	17.6
		28-Feb-2012	00903MOV (Sigma)		Yes	27.2
			1386561 (BASF)		No	16.6
						9.0
	BASF	18-May-2009	1386561 (Sigma)	open	No	86.6
		4-Feb-2010				38.9
		23-Sep-2010				42.2
		3-Nov-2011				25.7

Table 3. OP-KIT and BASF-OP2.0 comparisons. Opacities were measured in one instrument and immediately afterward in the second instrument at IIVS.

Sample Name	Testing Equipment	Mean Opacity	SD Opacity	Opacity Δ (OP-KIT, BASF-OP2.0)
Chlorhexidine	BASF-OP2.0	57.5	4.9	
	OP-KIT	52.0	5.7	-5.5
Dibenzoyl-L-tartaric acid	BASF-OP2.0	14.4	2.2	
	OP-KIT	17.3	2.6	2.9
Imidazole	BASF-OP2.0	64.4	1.7	
	OP-KIT	74.7	8.1	10.3
Ammonium nitrate	BASF-OP2.0	5.7	0.9	
	OP-KIT	4.7	2.1	-1.0
1-Naphthalene acetic acid	BASF-OP2.0	56.3	19.7	
	OP-KIT	68.0	23.6	11.7
Sodium oxalate	BASF-OP2.0	8.9	4.7	
	OP-KIT	13.7	3.5	4.8
4-Carboxybenzaldehyde	BASF-OP2.0	87.2	10.9	
	OP-KIT	97.7	12.7	10.5
Aluminum hydroxide	BASF-OP2.0	-0.3	0.1	
	OP-KIT	-2.7	0.6	-2.4
EDTA, di-potassium salt	BASF-OP2.0	-1.7	2.4	
	OP-KIT	-0.7	3.2	1

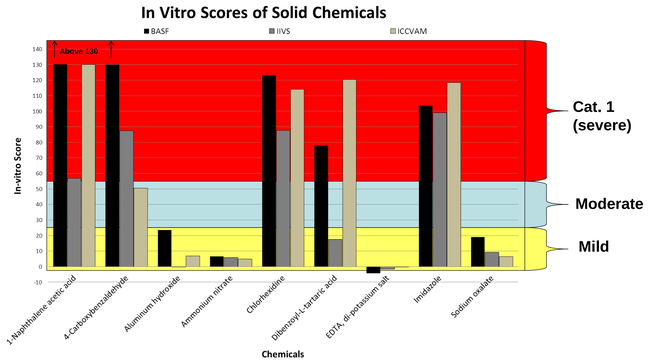


Figure 2. Comparison of In Vitro Scores between IIVS, BASF, and data published in ICCVAM BRD. Irritancy categories indicated by color: red- OECD Cat.1, blue- moderate, yellow- mild.

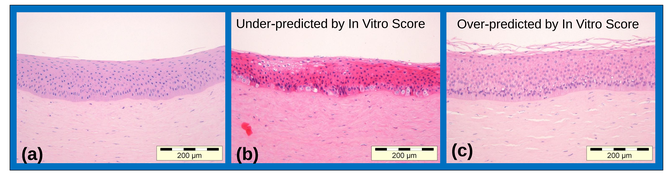


Figure 3. Histology images of (a) negative control, (b) sodium oxalate, and (c) 4-carboxybenzaldehyde treated corneas. (a) normal epithelium and upper stroma; (b) severe hyper-eosinophilia and multifocal vacuolation (full epithelial thickness); (c) protein precipitation and abnormal chromatin condensation (squamous and wing layers), cellular "edema" (full thickness), nuclear vacuolation (basal layer). Stroma appeared normal.

CONCLUSIONS

- Our data demonstrate high inter-laboratory reproducibility and correlation to previously published data.
- As compared to *in vivo* classifications, dibenzoyl-L-tartaric acid was under-predicted by BCOP at IIVS, and sodium oxalate was under-predicted by BCOP in both labs and by previous data. 4-carboxybenzaldehyde was over-predicted by BCOP at BASF, IIVS, and by previous data. The other chemicals were correctly classified.
- For chlorhexidine, using the same lot of chemical as BASF produced more similar results.
- For chlorhexidine and aluminum hydroxide closed chamber dose/rinse yielded higher In Vitro Scores than open chamber dose/rinse, but did not change irritancy category. Open chamber method allows more thorough rinsing of corneas.
- Histopathology improved understanding of eye irritation effects for some chemicals.
- BASF-OP2.0 opacitometer comparison showed an excellent correlation with data from the OP-KIT, and also showed less variability (smaller standard deviations- data not shown)

REFERENCES

- OECD TG 437 (2009). Bovine Corneal Opacity and Permeability Test Method for Identifying Ocular Corrosives and Severe Irritants.
- ICCVAM BRD (2006). In Vitro Test Methods for Detecting Ocular Corrosives and Severe Irritants.
- Schrage *et al.* (2011). The Bovine Corneal Opacity and Permeability Test in Routine Ocular Irritation Testing and Its Improvement Within the Limits of OECD Test Guideline 437. **ATLA** 39, 37-53.