AATEX 3, 137–145 (1995)

First Phase Inter-Laboratory Validation of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients: (2) Evaluation of Chorioallantoic Membrane (CAM) Tests

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SUMMARY

The chorioallantoic membrane (CAM) test using fertile hen's egg was evaluated in five laboratories as an alternative method to predict eye irritation of cosmetic ingredients. Nine surfactants and physiological saline were used as coded samples. Test procedures were controlled under the same standard operating procedure (SOP) among participants. CAM responses to the chemials was measured using two systems; the macroscopic observation method (HET-CAM method) and trypan blue staining method.

The rank correlation coefficients between the lead laboratory and the other four laboratories were 0.77–0.99 for HET-CAM method and 0.88–0.93 for trypan blue staining method, respectively. The inter-laboratory variations of both methods were relatively small except for cases of non irritating samples. The correlation coefficient and the rank correlation coefficient between the HET-CAM scores and the maximum total scores of Draize eye irritation tests were 0.75 and 0.94, respectively. Those between the amount of trypan blue staining and the maximum total scores of Draize eye irritation tests were 0.95 and 0.91, respectively. When we compared these *in vitro* results with individual scores in the Draize eye irritation test, HET-CAM results showed a good correlation to the changes in the conjunctiva. On the other hand, trypan blue staining method showed a good correlation to those in cornea.

From these results, we conclude that the HET-CAM method and trypan blue staining method using CAM are promising alternative methods to the Draize eye irritation test. Further evaluation of these methods using a wider range of cosmetic ingredients is under way.

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Key Words: CAM, Validation study, Draize eye irritation test, Alternatives, Surfactant, HET-CAM, Trypan blue staining, *In vitro*

INTRODUCTION

The HET-CAM method, first reported by Luepke[1], has been extensively studied and

validated in various research facilities. The basic principle of this method is that macroscopic changes in the CAM, such as hyperaemia, haemorrhage and coagulation, follow treatment with test chemicals. Therefore, this has an advantage of being able to evaluate changes in blood vessels, which is not possible by most of the other *in vitro* methods. Sterzel et al.[2], Kalweit et al.[3,4], Spielmann et al.[5,6], Blein et al.[7], Bagley et al.[8], De Silva et al.[9] and Hagino et al.[10,11] showed a useful correlations between the HET-CAM test and *in vivo* eye irritation. On the other hand, Reinhardt et al.[12] and Lawrence et al.[13] reported that this test was not fully predictive for eye irritation. As a whole, it has been proposed that this method is useful as a screening test or as a part of a battery to predict the eye irritation of chemicals.

The trypan blue staining method was developed as an objective evaluation technique to overcome disadvantages arising from the lack of objectivity and quantitativeness in the HET-CAM method[10,11]. The basic principle of this method is to examine the injurious effect of chemicals by measuring the amount of trypan blue adsorbed with a CAM as an end point. Trypan blue staining, widely used for measuring cell viability, detects destruction and denaturation of membranes and the results are supposed to correlate to the corneal score of Draize eye irritation tests. This procedure can be performed effectively using the same eggs after macroscopic observation of CAM in HET-CAM method.

We conducted a first phase inter-laboratory evaluation of the HET-CAM method and the trypan blue staining method for predicting the eye irritation of cosmetic ingredients. It was performed using nine surfactants and isotonic sodium chloride solution (physioloical saline) as a negative control by five independent laboratories under the same SOP. This is a part of the MHW project entitled "Studies on the test methods to evaluate the safety of new ingredients of cosmetics."

MATERIALS AND METHODS

Materials

The test substances used in this project were four nonionic surfactants, four anionic surfactants, one cationic surfactant and physiological saline, as shown in Table I. They were from Japanese standards of cosmetic ingredients and supplied from Japan Cosmetic Industry Assoc. (JCIA) to National Institute of Health Sciences (NIHS). the substances coded in NIHS were sent to each laboratory. Each surfactant was dissolved or suspended in distilled water at 10% (W/V) concentration and used for *in vivo* and *in vitro* test. All other reagents were obtained commercially and were of the highest grade available.

In vivo test

The *in vivo* test was performed using conventional Draize eye irritation test methods[14] and separately reported by Ohno et al[15].

Table I. List of the test substances

Sample No.	Test substance	Abbreviation	Classification
S-1	Isotonic Sodium Chloride Solution	Physiological saline	
S-2	Polyoxyethylene Hydrogenated Castor Oil (60 E.O.)	POE hydrogenated castor oil	Nonionic
S-3	Polyoxyethylene Sorbitan Monolaurate (20 E.O.)	Tween 20	Nonionic
S-4	Polyethyleneglycol Monolaurate (10 E.O.)	PEG monolaurate	Nonionic
S-5	Sodium N-Lauroyl Sarcosinate (30% solution)	Lauroyl sarcosinate	Anionic
S-6	Sodium Hydrogenated Tallow L-glutamate	HT-glutamate	Anionic
S-7	Sodium Lauryl Sulfate	SLS	Anionic
S-8	Sodium Polyoxyethylene Laurylether Sulfate (2 E.O.: 27% solution)	POE laurylether sulfate	Anionic
S-9	Polyoxyethylene Octylphenylether (10 E.O.)	Triton X-100	Nonionic
S-10	Benzalkonium Chloride	Benzalkonium chloride	Cationic

The surfactants (from S-2 to S-10) were dissolved/suspended in distilled water at 10% (w/v) concentration and used for *in vivo* and *in vitro* test.

In vitro test

The test was performed using the coded samples and the same SOP by five laboratories (shown as Laboratory A, B, C, D and E). The technology transfer to each participated laboratory was done by lead laboratory A. The application procedure of the test solution was basically the same as the original method developed by Luepke[1], except for the use of the silicone ring to define precisely the application area[11]. The magnitude of CAM injury after treatment of chemicals was measured by two systems; HET-CAM method (macroscopic observation) and trypan blue staining method (measurement of trypan blue adsorbed with the treated site).

Eggs and incubation

Fertile eggs of White Leghorn chickens were obtained from Nippon Bio-supp. Center Co., Ltd except for laboratory D, which obtained them from Shimizu Laboratory Supply Co., Ltd. Eggs were incubated for ten days in an incubator (P-008 Type, Showa Incubator Laboratory) at 37.6°C under a relative humidity of about 70% and were turned automatically once per hour.

Application of test chemicals

Four eggs were used for each sample. On day 10 of incubation, a portion of egg shell above the air-space was removed. A drop of water was placed on the shell membrane to avoid capillary bleeding[16], then the CAM was exposed carefully with the aid of forceps. A silicon ring with inner diameter of 18 mm was placed on the CAM. Two hundred μl of test solution was applied inside of the ring on the CAM and washed off with a gentle flow of distilled water after 20 seconds.

HET-CAM method[1]

Each CAM was examined and graded macroscopically for hyperaemia, haemorrhage and coagulation at 0.5, 2 and 5 minutes after treatment with test solution. The score was assigned on the basis of the time of onset of each effect (Table II).

Table II. Scoring schema of the HET-CAM test

Effect	Score		
Time - - -	0.5	2	5 (min.)
Hyperaemia	5	3	1
Haemorrhage	7	5	3
Coagulation	9	7	5

The scoring scheme was the same as that used by Luepke.

Trypan blue staining method[10,11]

Immediately after the final macroscopic observation, the CAM was treated with 0.5 ml of 0.1% trypan blue in phosphate-buffered saline (PBS) (pH 7.4) for 1 minute. Excess pigment on the CAM was rinsed off with distilled water for 20 seconds. The dyed CAM was excised and the adsorbed trypan blue was extracted with 3 ml of formamide. The absorbance of the extract was measured spectrophotometrically at 595 nm (scanned from 500 to 700 nm).

RESULTS

1. HET-CAM method

The results of the HET-CAM tests at the five laboratories are presented in Table III. The rank correlation coefficients between the lead laboratory A and the other four laboratories were 0.988 for B, 0.964 for C, 0.861 for D and 0.770 for E, respectively. The mean of the coefficients of variation was 0.50 for ten samples and 0.31 for nine samples except for physiological saline (S-1).

Table IV shows results obtained from the Draize eye irritation test. The first parameter was maximum average of three rabbits calculated at each observation time point. The second parameter was score at 24 hr after application. The third parameter was area ratio under the curve, which stands for ratio (%) of the area under the line connecting scores at each observation period to those based on theoretical maximum of Draize total score until 7 days after application. These parameters were obtained for each evaluated tissue of the rabbit eye (cornea, iris and conjunctiva).

Table III. Results of the HET-CAM test at five laboratories

Sample No.	A	B	C*		D	E	Mean±S.D.	Coefficient of variance
S-1	0.00	0.00	0.00	(6.50)	1.75	0.00	0.35±0.78	2.24
S-2	0.75	1.25	2.75	(9.25)	2.50	0.00	1.45±1.16	0.80
S-3	1.00	5.50	3.00	(11.00)	9.50	7.50	5.30±3.40	0.64
S-4	4.75	6.75	2.25	(15.75)	10.00	11.00	6.95±3.63	0.52
S-5	11.50	9.50	9.50	(17.25)	12.00	9.00	10.30±1.35	0.13
S-6	11.00	7.00	7.50	(11.75)	12.00	10.50	9.60±2.22	0.23
S-7	12.00	11.00	10.50	(17.00)	11.00	10.60	11.02±0.59	0.05
S-8	10.00	9.00	6.25	(14.00)	6.75	8.75	8.15±1.59	0.19
S-9	12.00	11.00	10.50	(15.75)	10.50	9.50	10.70±0.91	0.08
S-10	14.75	11.50	13.25	(15.50)	15.75	14.00	13.85±1.61	0.12
R**	-	0.988	0.964		0.861	0.770		

*: The results of laboratory C were of the retest after breaking the sample codes, because of the higher scores indicated in parenthesis.
**: R stands for the rank correlation coefficient to the scores of laboratory A.

Table IV. Results of the Draize eye irritation test on the ten samples

Sample No.	Maximum score				24 hr score				Area ratio under the curve*			
	Total	Cornea	Iris	Conjunctivae	Total	Cornea	Iris	Conjunctivae	Total	Cornea	Iris	Conjunctivae
S-1	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S-2	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0
S-3	0.7 (1)**	0.0	0.0	0.7 (1)	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1
S-4	3.3 (1)	0.0	0.0	3.3 (1)	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.2
S-5	10.3 (48)	8.3 (48)	0.0	8.0 (1,4)	8.3	5.0	0.0	3.3	3.4	1.9	0.0	1.5
S-6	26.7 (24)	16.7 (24,48,72)	1.7 (72)	12.0 (4)	26.7	16.7	0.0	10.0	14.9	10.7	0.8	3.5
S-7	15.0 (4)	8.3 (48,72)	0.0	10.0 (4)	14.7	6.7	0.0	8.0	7.1	4.2	0.0	3.0
S-8	10.0 (4)	3.3 (48)	0.0	10.0 (4)	2.7	0.0	0.0	2.7	2.0	0.7	0.0	1.4
S-9	41.3 (72)	30.0 (72)	5.0 (168)	10.0 (48)	24.7	15.0	1.7	8.0	26.9	18.4	2.3	6.3
S-10	78.0 (24)	66.7 (24)	5.0 (96-168)	14.7 (96)	78.0	66.7	0.0	11.3	57.3	43.9	2.5	10.9

*: The area ratio under the curve means the ratio (%) of the area under the line connecting scores at each observation period to those based on theoretical maximum of Draize total score until 7 days after treatment.
**: These values in parenthesis are the time (hour) when the scores became maximum.

Table V. Correlation between the HET-CAM test and the Draize eye irritation test*

Parameter of the Draize eye irritation test	Regression formula	Correlation coefficient	Rank correlation coefficient
Total score			
maximum	y=4.284x-14.75	0.748	0.936
24 hour	y=3.968x-15.31	0.706	0.885
Area ratio under the curve	y=2.902x-11.35	0.681	0.936
Corneal score			
maximum	y=3.419x-13.23	0.700	0.900
24 hour	y=3.068x-12.82	0.644	0.855
Area ratio under the curve	y=2.142x-8.66	0.660	0.909
Iris score			
maximum	y=0.287x-1.06	0.594	0.664
24 hour	y=0.030x-0.06	0.239	0.515
Area ratio under the curve	y=0.140x-0.52	0.601	0.673
Conjunctivae score			
maximum	y=1.150x-2.06	0.918	0.864
24 hour	y=0.870x-2.43	0.825	0.894
Area ratio under the curve	y=0.625x-2.17	0.766	0.936

*: The values were caluculated by the mean of the HET-CAM test data of five laboratories from Table III and the Draize eye irritation test data from Table IV.

Table V shows the regression formula, the correlation coefficient and the rank correlation coefficient between the HET-CAM test scores and various parameters of the Draize eye irritation test. The correlation coefficients between the HET-CAM test scores and the three parameters of total score were 0.681–0.748. The rank correlation coefficients between them were 0.885–0.936. The correlation to conjunctivae score was higher than to corneal or iris score, and the correlation coefficients were 0.766–0.918.

The relationship between the HET-CAM test score and maximum total score of the Draize eye irritation test is shown in Fig. 1. The classification of eye irritation potencies of ten samples were analyzed using this regression line. Test substances with more than 15 of Draize maximum total score were considered to be positive irritants. Accordingly, the *in vitro* cut-off point of 6.94 was calculated from the regression formula y=4.284x–14.75 for the maximum total score 15. Two (Lauroyl sarcosinate (S-5), POE laurylether sulfate (S-8)) of ten samples were classified as false positives. However, there was no false negatives.

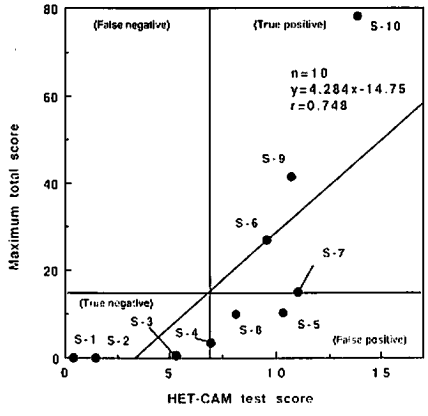

Figure 1. The relationship between the HET-CAM test score and maximum total score of the Draize eye irritation test. The test chemicals are numbered in Table I.

2. Trypan blue staining method

The results of the trypan blue staining tests at the five laboratories are shown in Table VI. the rank correlation coefficients between the lead laboratory A and the other four laboratories were 0.903 for B, 0.879 for C, 0.927 for D and 0.891 for E, respectively. The mean of the coefficients of variation was 0.45 for ten samples and 0.39 for nine samples except for physiological saline (S-1).

Table VII shows the regression formula, the correlation coefficient and the rank correlation coefficient between the amount of the trypan blue adsorbed with the CAM and the various basic scores of the Draize eye irritation test. The amount of trypan blue showed a good correlation with corneal scores in the Draize eye irritation test; the correlation coefficients were 0.962 for maximum score, 0.963 for 24 hr score and 0.953 for the area

Table VI. Results of trypan blue staining test at the five laboratories*

Sample No.	A	B	C**	D	E**		Mean±S.D.	Coefficient of variance
S-1	0.41±0.82	1.98±2.65	0.20±0.32	0.28±0.38	1.19±1.51	(0.00±0.00)	0.81±0.76	0.94
S-2	0.99±0.69	3.72±3.22	0.10±0.20	2.54±0.69	2.17±2.57	(0.02±0.05)	1.90±1.40	0.74
S-3	5.06±1.62	4.23±1.80	1.85±0.13	5.25±0.82	3.75±1.13	(0.34±0.67)	4.03±1.36	0.34
S-4	6.03±1.45	3.76±3.16	4.24±4.23	8.20±4.66	3.76±0.92	(2.57±1.75)	5.20±1.92	0.37
S-5	13.47±3.83	12.89±3.20	2.45±3.31	10.41±2.24	11.98±3.84	(6.17±2.51)	10.24±4.51	0.44
S-6	15.66±1.55	12.45±2.55	8.19±4.02	8.53±1.14	8.68±3.40	(5.44±0.68)	10.70±3.27	0.31
S-7	13.83±1.94	11.48±7.41	7.14±6.47	6.88±3.33	11.11±4.67	(5.70±1.12)	10.09±3.00	0.30
S-8	16.52±3.06	11.83±2.23	4.55±5.58	14.34±3.36	10.81±3.05	(2.40±1.74)	11.61±4.53	0.39
S-9	23.20±3.43	18.04±8.81	6.87±4.61	11.98±1.75	14.47±1.51	(5.95±0.48)	14.91±6.16	0.41
S-10	44.19±7.87	55.05±12.30	52.07±29.28	34.67±8.77	30.83±10.27	(9.28±1.44)	43.36±10.56	0.24
R+	—	0.903	0.879	0.927	0.891			

*: The values at five laboratories are the mean (nmol)±standard deviation of the four eggs.
**: The results of laboratory C and E were of the retest after breaking the sample codes, because of the deviation from SOP at laboratory C and the lower values indicated in parenthesis at laboratory E, respectively.
+: R stands for the rank correlation coefficient to the values of laboratory A.

Table VII. Correlation between the trypan blue staining test and the Draize eye irritation test*

Parameter of the Draize eye irritation test	Regression formula	Correlation coefficient	Rank correlation coefficient
Total score			
maximum	y=1.940x-3.37	0.954	0.912
24 hour	y=1.914x-6.09	0.960	0.861
Area ratio under the curve	y=1.436x-5.02	0.950	0.912
Corneal score			
maximum	y=1.667x-5.48	0.962	0.894
24 hour	y=1.629x-7.38	0.963	0.758
Area ratio under the curve	y=1.097x-4.40	0.953	0.885
Iris score			
maximum	y=0.133x-0.33	0.777	0.779
24 hour	y=0.0046x+0.12	0.105	0.576
Area ratio under the curve	y=0.066x-0.19	0.802	0.782
Conjunctivae score			
maximum	y=0.344x+2.99	0.774	0.912
24 hour	y=0.280x+1.17	0.749	0.839
Area ratio under the curve	y=0.273x-0.39	0.943	0.912

*: The values were caluculated by the mean of the trypan blue staining test data of five laboratories from Table V and the Draize eye irritation test data from Table IV.

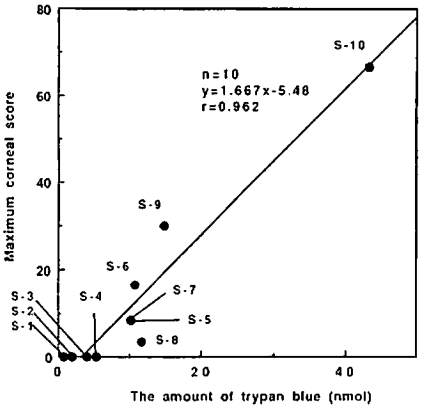

Figure 2. The relationship between the amount of trypan blue and the maximum corneal score of Draize eye irritation test. The test chemicals are numbered in Table 1.

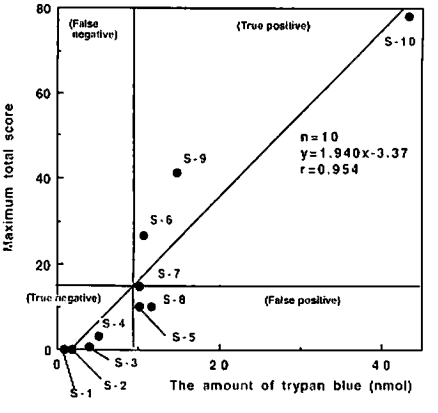

Figure 3. The relationship between the amount of trypan blue and the maximum total score of the Draize eye irritation test. The test chemicals are numbered in Table 1.

ratio under the curve. The correlation to the total score was also high, and the correlation coefficients were 0.950–0.960 for the three parameters. When compared with the iris and the conjunctivae score, the correlation coefficients to the area ratio under the curve were 0.802 and 0.943, respectively, which were higher than those of maximum and 24 hr scores. Fig. 2 shows the relationship between the amount of trypan blue adsorbed with the CAM and maximum corneal score of the Draize eye irritation test as the most correlative example.

The relationship between the amount of trypan blue adsorbed with the CAM and maximum total score is shown in Fig. 3. The classification of eye irritation potencies of ten samples were tried using this regression line. The calculation was performed in the same way as for the HET-CAM test. The *in vitro* cut-off point was 9.47 calculated from the regression formula $y=1.940x-3.37$ for the maximum total score 15. Two (Lauroyl sarcosinate (S-5), POE laurylether sulfate (S-8)) of ten samples were classified as false positives. However, there was no false negatives.

DISCUSSION

The HET-CAM test, proposed by Luepke[1], has been studied by many investigators. Most of them concluded that this method was rapid, inexpensive and effective as an alternative to the eye irritation test. On the other hand, it has been pointed out that the scoring system has disadvantages arising from the lack of objectivity and quantitativeness. Kalwait et al.[3,4] showed a problem concerning its reproducibility in an inter-laboratory study. Luepke and Wallat[17] reported that this method required an experienced investigator for proper judgment. Therefore, the technology transfer and the detailed SOP are very important for this method.

The HET-CAM scores first obtained at laboratory C, shown in parenthesis in Table III, were high compared to the other four laboratories, even in the case of physiological saline. Therefore, a retest was carried out after breaking the sample codes under the supervision of lead laboratory A. At that time, care was taken to avoid hyperaemia and/or haemorrhage arising from a strong stream of distilled water when irrigating the sample from the surface of CAM. As a result of the retest, the values obtained at laboratory C were in the range of the other four laboratories. It indicates that test samples should be more gently irrigated from a CAM if the application of physiological saline (S-1) causes hyperaemia and/or haemorrhage on the CAM. The variances of HET-CAM scores among five laboratories, including the data after retest and excluding the data before the retest, seemed to be small except for cases of non irritating samples, and the rank correlation coefficients between the lead laboratory A and the other four laboratories were relatively high. These results indicate that the inter-laboratory variance of the methods may be small if participants are well trained according to the SOP.

Trypan blue staining method has been developed as an objective evaluation technique of CAM damage. Since the measurement is simple and quantitative, a good inter-laboratory variance was expected. However, the amount of trypan blue first obtained at laboratory C and E were very different from the other three laboratories. The former seemed to be caused by deviation from the SOP (data not shown). The latter seemed to be caused by the wrong reagent, which was trypan blue. After retests were carried out at both laboratories after breaking the sample code, the values were improved. The variances of the values among five laboratories, using the data after retest, seemed to be small except for cases of non irritating samples, and the rank correlation coefficients between the lead laboratory A and other four laboratories were relatively high. These results indicate that the trypan blue staining method may have good inter-laboratory reproducibility.

When the HET-CAM score was compared with total score of the Draize eye irritation test, the results did not fit well with a simple regression line (Fig. 1), indicating linear regression may not be appropriate for the HET-CAM method. However, the rank correlation were high and the coefficients were 0.885–0.936, suggesting that the HET-CAM method might be able to be utilized to evaluate the eye irritation potential by the comparison with reference substances.

Scoring in the Draize eye irritation test depends on the individual changes in cornea, iris and conjunctivae observed after application of test substances. To identify the most correlative parameter to the *in vitro* results, we compared the *in vitro* results with the individual scores as well as the total of those scores. The individual scores were represented by maximum score and 24 hr score as conventional parameter, and the area ratio under the curve which was calculated as a parameter included the factor of progression, persistency and recovery in eye irritation. The result of the HET-CAM test was more correlative to the maximum score of conjunctiva

than the other parameters (r=0.918).

The amounts of trypan blue adsorbed with the CAM were compared with the corneal scores obtained from Draize eye irritation test. The correlation coefficients with simple linear regression were high (r=0.953–0.963) for those parameters of cornea, suggesting that this method may be suitable for predicting corneal injury. The corneal score contributes about 70% to the total score in the Draize test. Therefore, it is not surprising that there was also a good correlation (r=0.954–0.960) between the values from the trypan blue staining and the total score of the Draize test.

Since the HET-CAM test and the trypan blue staining test were most correlative to conjunctivae score and corneal score in the Draize eye irritation test, respectively, it provides the hopeful indication that these two methods combined together may constitute a useful alternative method to predict the eye irritation.

Predictability of HET-CAM method and trypan blue staining method were evaluated by using the regression line and 15 as the discriminative value in the Draize maximum total score. Both methods had no false negatives. However, Lauroyl sarcosinate (S-5) and POE laurylether sulfate (S-8) were classified as false positive by both. The deviations of Lauroyl sarcosinate (S-5) and POE laurylether sulfate (S-8) results from the regression line in the HET-CAM method seemed bigger than those in trypan blue staining method. The reason why they deviated from the regression line is uncertain. As a whole, the results suggest that both methods may have a useful predictability to discriminate between positive and negative eye irritants.

We conclude that the HET-CAM method and trypan blue staining mehod using CAM are promising alternative methods to the eye irritation test for surfactants. The second phase validation of these methods using a wider of cosmetic ingredient is under way.

ACKNOWLEDGMENT

This study was partly supported by Research Grant for Health Sciences from Ministry of Health and Welfare. The authors thank many individuals who participated in this project and aided the preparation of this manuscript.
(Received: July 14, 1995; accepted: October 11, 1995)

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