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First Phase Inter-Laboratory Validation of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients: (1) Overview, Organization and Results of the Validation Study

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SUMMARY

Preliminrary validation studies on twelve methods proposed as alternatives to the Draize eye irritation test using nine coded surfactants and physiological saline solution were conducted by twenty laboratories including a National Health Institute, universities,

kit suppliers, and cosmetic companies. The results indicated that 1) intra- and inter-laboratory variations were generally small excepting the study of hemolysis by Tween 20, 2) rank order of the cytotoxicity potentials of Triton X-100 by cultured cell methods with serum differed greatly from those without serum, 3) correlation coefficients between the results of *in vitro* method and *in vivo* Draize test are higher than 0.8 for HET-CAM-trypan blue staining method and cultured cell methods with serum. Rank order correlation coefficients between them were higher than 0.9, 4) correlation coefficients among cultured cell methods using serum were also high (r=0.92–0.99). These preliminary results suggest that several of the *in vitro* methods evaluated in this report may be useful for the prediction of eye irritancy potential of cosmetic ingredients. However, further validation studies using a wider range of chemicals are necessary.

INTRODUCTION

The Draize rabbit eye-irritation test (Draize test)[1] has been widely used to evaluate the potential eye irritancy of a wide range of chemicals including pharmaceuticals, cosmetics and their raw materials. However, the Draize test has recently been criticized from the viewpoint of animal welfare. In order to replace this technique and to minimize the use of animals in such experiments, investigators have proposed various methods as alternatives to the Draize test for predicting the potential of chemicals to cause ocular irritation[2,3].

In 1991, the Japanese Ministry of Health and Welfare began a "Study on test methods to evaluate the safety of cosmetics containing new ingredients"[4]. The objective of this project was to investigate the methods proposed as alternatives to the Draize test and to assess the possibility of replacing the *in vivo* eye irritation test with *in vitro* methods. We reviewed literature, discussed 16 methods in detail, and selected twelve methods for inter-laboratory validation studies. Selection was based on the scientific basis of the methods, relevance to the Draize test, expenses, our own experiences, and the potential of these methods in Japan including ethical considerations. The methods selected were:

1) HET-CAM macroscopic observation method (HET-CAM)[5],
2) HET-CAM-trypan blue staining method (CAM-TB)[6,7],
3) Hemolysis method (RBC)[8],
4) Hemoglobin denaturation method (Hb)[9,10],
5) Artificial skin model SKIN2™ (ZK1100 model)[11],
6) Artificial skin model MATREX™[12,13],
7) Normal rabbit corneal epithelial cells (CornePack)[14],
8) SIRC-crystal violet method (SIRC-CV)[15],
9) SIRC-neutral red methods (SIRC-NR)[15],
10) HeLa-MTT methods[16],
11) CHL-crystal violet methods (CHL-CV)[17],
12) EYTEX®[18].

This paper summarizes the study, the organization of the researchers and institutions involved, and the results of the first phase validation tests of *in vitro* alternative methods. We also describe the results of Draize tests using the same lot of test substances, which were performed out of necessity in order to evaluate the results of the *in vitro* methods. Data obtained by individual methods have been reported elsewhere[19–27].

METHODS

Organization of the validation

As shown in Figure 1, we created several committees in order to conduct this inter-laboratory validation study effectively. The organization consisted of 4 major committees and 9 working groups for 12 test methods. The *validation management committee*, consisting

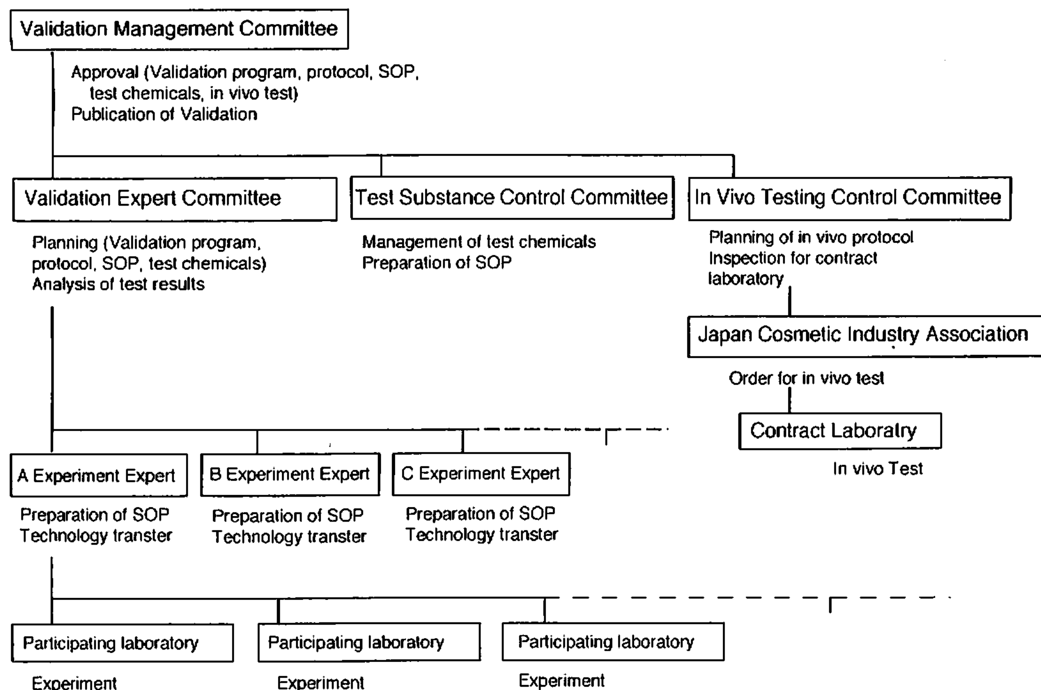


Figure 1. Organization for the primary validation

of 5 scientists, one administrator, and several scientists acting as secretaries, dealt with general matters, final approval of the validation program, and publication of the results. The *validation expert committee*, consisting of representatives from the committees indicated below, experts of statistics and of each nominated alternative method, and secretaries, dealt with planning and control of the tests and analysis of the results. As study directors of the working groups for each test method, these experts were also responsible for drafting standard operation procedure (SOP), transferring technology, controlling experiments, collecting data, and performing preliminary data analysis. The SOP drafts were discussed by the *validation expert committee* and then sent to the *validation management committee* for further discussion and final approval. Both the validation management committee and the validation expert committee were chaired by Dr. Takanaka. The *test substances control committee* dealt with the management of test chemicals, including preparation of SOP of related matters, storage, specification, coding, and distribution of test substances. The *in vivo test management committee*, consisting of toxicologists, a pathologist, and representatives of the Japan Cosmetic Industry Association (JCIA), arranged the Draize test, selected the contract laboratory for the Draize test, checked the SOPs of this laboratory and monitored the experiments. JCIA asked the contract laboratory (Japan Seigiken Research Center Co. Ltd.) to conduct the Draize test according to the plan approved by the *validation management committee.*

Methods tested and participating organizations

Methods investigated are listed in Table I. Twenty laboratories including a National Health Institute, universities, kit suppliers, and JCIA member companies participated in the study (Table II). Each method, except for MATREX^{TM}, was evaluated by at least five laboratories in order to obtain information about inter-laboratory variation of the results.

Table I. Test methods and participants

Methods	A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	total
Chorioallantoic membrane (CAM)																		
Macroscopic observation	●	●			●		●					●						5
Trypan blue staining	●	●			●		●					●						5
Red blood cell (RBC)																		
Hemolysis of RBC	●		●	●			●	●				●	●	●		●		9
Hemoglobin denaturation	●		●				●	●				●	●	●		●		8
Artificial models of skin corium																		
SKIN2	●			●							●	●				●	●	6
TESTSKIN (MATREX)					●	●											●	3
Normal rabbit corneal epithelial cells																		
CornePack	●									●		●		●	●		●	6
Established cell line of rabbit corneal origin																		
SIRC-CV	●	●	●			●						●			●			6
SIRC-NR	●	●	●	●			●					●			●			7
Established cell line of mammalian origin																		
HeLa-MTT	●		●	●		●			●	●		●	●					8
CHL-CV	●		●				●		●	●		●	●					7
EYTEX			●		●			●								●	●	5
Total	10	4	7	4	4	3	6	3	2	3	1	10	4	3	3	4	4	75

Q: Indicate the test kit suppliers.

Table II. List of the participated organizations

Administrative Organizations	Japan Cosmetic Industry Association
Ministry of Health and Welfare	Shiseido Co. Ltd.
National Institute of Health Sciences	Pola Corp.
	Kanebo, Ltd.
Universities	Kose Corp.
Yokohama-City University	Lion Corp.
Showa University	Kao Corp.
	Sunstar Inc.
Kit suppliers	Oppen Cosmetics Co. Ltd.
Oriental Yeast Co., Ltd.	Noevir Co., Ltd.
Kurabo Industries Ltd.	Kaminomoto Co., Ltd.
Invitro International Japan Ltd.	Procter & Gamble Far East, Inc.
Toyobo Co., Ltd.	Nippon Menard Cosmetics Co., Ltd.
	Yakult Honsha Co., Ltd.
	Ajinomoto Co., Inc.

Table III. List of the test chemicals and their expected eye irritatancy on rabbits

Sample number	Test Chemicals	Abbreviation	Classification	Expected Eye Irritation potency#
S-1	Isotonic Sodium Chloride Solution	Physiological Saline	-	Non##
S-2	Polyoxyethylene Hydrogenated Caster Oil (60 E.O.)	POE hydrogenated caster oil	Nonionic	Non
S-3	Polyoxyethylene Sorbitan Monolaurate (20 E.O.)	Tween 20	Nonionic	Slight
S-4	Polyethylene glycol Monolaurate (10 E.O.)	PEG monolaurate	Nonionic	Slight
S-5	Sodium N-Lauroyl Sarcosinate (30% solution)	Lauroyl sarcosinate	Anionic	Mild
S-6	Sodium Hydrogenated Tallow L-Glutamate	HT-glutamate	Anionic	Mild
S-7	Sodium Lauryl Sulfate	SLS	Anionic	Moderate
S-8	Sodium Polyoxyethylene Laurylether Sulfate (2E.O.) (27% solution)	POE Laurylether sulfate	Anionic	Moderate
S-9	Polyoxyethylene Octylphenylether (10 E.O.)	Triton X-100	Nonionic	Severe
S-10	Benzalkonium Chloride	Benzalkonium chloride	Cationic	Severe

#: Expected eye irritations on rabbits were estimated for 10% (w/v) aqueous solution (100ul) from the data accumulated in cosmetic companies which belong to Japan Cosmetic Industry Association.
##: Classified by Draize scores (non : 0.0-0.5, slight : 0.5-15, mild : 15-25, moderate : 25-50, severe : 50-110)

Most of the participating laboratories, except for the test kit suppliers. took part at least three investigations (Table I).

Test substances

The 10 test substances used in this study are listed in Table III. They comprised one cationic surfactant (benzalkonium chloride), four anionic surfactants (Lauroyl sarcosinate, HT-glutamate, SLS, POE laurylether sulfate), four nonionic surfactants (POE hydrogenated castor oil, Tween 20, PEG monolaurate, Triton X-100) and isotonic sodium chloride solution (physiological saline). These substances were selected to cover each category of eye irritation potential. Each substance met Japanese Standards for Cosmetic Ingredients or for Japanese Pharmacopoeia and were supplied from JCIA to the National Institute of Health Sciences (NIHS).

Surfactants were selected because they are the most widely-used chemicals in cosmetics and include every category of eye irritation potential. However, surfactants constitute only a part of cosmetic ingredients in use. Thus, we consider this project to be a preliminary investigation. Further validation studies using a wider range of cosmetic ingredients are necessary.

The test substances were subdivided, coded, and supplied to each participating laboratory by the *test substances control committee*. This work was primarily done by one NIHS researcher who did not participate in any of the individual tests in order to obtain objective information about intra- and interlaboratory variation. However, it was necessary to distribute the list of the test substances to the participants without code to ensure proper handling and disposal of the test substances.

Procedure of the tests

Test procedures in each participating laboratory were strictly controlled by common SOPs. Technology transfers were conducted by the study director of each test and/or kit suppliers. Minor modifications of SOPs were made in some tests after initial trials. Much of the content of these SOPs has been described in other papers[16-24].

Participants were asked to mimic some aspects of GLP procedures. Participants heard a lecture from a GLP inspector, experiments were conducted according to SOPs, quality of the test substances was controlled by the *test substances control committee*, documentation was required at key steps of the experiment, and all documents were checked by the study director of each test method and stored according to GLP procedures.

Calculation of results

Results were calculated according to the methods of each test[19-27]. In the case of the hemolysis test and other tests using cultured cells, the concentration of each substance required to inhibit each endpoint by 50% (EC_{50}) was calculated from dose response curves obtained by a computer. If the curve did not fit the original data well, EC_{50} was obtained from the straight line connecting the nearest two points spanning 50%.

Draize eye irritation test

We also conducted the Draize eye irritation test[1] using the same lot of test substances in order to avoid variation of the results caused by lot difference. This test was conducted by Japan Seigiken Research Center Co. Ltd. according to GLP standards.

100 μl aqueous solution of the test substances (10%) was applied to the right eyes of male New Zealand White rabbits (2.30–2.98 kg, 13 weeks). Left eyes were left untreated as a control. Eyes were observed at 1 hour, 4 hours, and every 24 hours after the application for 7 days. Three rabbits were used for each test substance.

For the purpose of comparing *in vitro* data and *in vivo* data, several parameters were calculated from the results of the Draize test. The first parameter was a maximum average Draize score, the maximum of average scores

calculated at each observation of three rabbits after the application of test substances. The second parameter was scores at 24 hours after application. The third was area under the curve (AUC), which was obtained by calculating the area under the curve connecting scores at each observation until 7 days after application of test substances. These parameters were calculated from scores for each part of rabbit eye (cornea, iris, conjunctivae) and also from the sum of these scores.

RESULTS AND DISCUSSION

1. Results of the Draize test

The results of the Draize test are shown in Tables IV and V. maximal average Draize score, scores at 24 hours after application, and AUC were calculated for cornea, iris, conjunctivae and sum of these scores (total average score). These values were used for comparison with data obtained by testing *in vitro* alternative methods.

Figure 2 indicates time-dependent changes of total average Draize scores (total scores). Scores for most of the test substances peaked

Table IV. Results of Draize eye irritation test (1)

Sample	Maximum average Draize scores (MAS)			
number	Total#	Cornea	Iris	Conjunctiva
S-1	0.0	0.0	0.0	0.0
S-2	0.0	0.0	0.0	0.0
S-3	0.7 (1)	0.0	0.0	0.7 (1)
S-4	3.3 (1)	0.0	0.0	3.3 (1)
S-5	10.3 (48)	8.3 (48)	0.0	8.0 (1,4)
S-6	26.7 (24)	16.7 (24,48,72)	1.7 (72)	12.0 (4)
S-7	15.0 (4)	8.3 (48, 72)	0.0	10.0 (4)
S-8	10 (4)	3.3 (48)	0.0	10.0 (4)
S-9	41.3 (72)	30.0 (72)	5.0 (168)	10.0 (48)
S-10	78.0 (24)	66.7 (24)	5.0 (96-168)	14.7 (96)

These values indicate the maximal average Draize scores and numerals in parenthesis indicates the time (hour) when the scores became maximum.
#: indicates the sum of the scores of cornea, iris, and conjunctiva.

Table V. Results of Draize eye irritation test (2)

Sample	Scores of 24 hr after				AUC (%)*			
number	total#	cornea	iris	conjunctiva	total#	cornea	iris	conjunctiva
S-1	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
S-3	0.0	0.0	0.0	0.0	0.1	0.0	0.0	0.1
S-4	0.0	0.0	0.0	0.0	0.2	0.0	0.0	0.2
S-5	8.3	5.0	0.0	3.3	3.4	1.9	0.0	1.5
S-6	26.7	16.7	0.0	10.0	14.9	10.7	0.8	3.5
S-7	14.7	6.7	0.0	8.0	7.1	4.2	0.0	3.0
S-8	2.7	0.0	0.0	2.7	2.0	0.7	0.0	1.4
S-9	24.7	15.0	1.7	8.0	26.9	18.4	2.3	6.3
S-10	78.0	66.7	0.0	11.3	57.3	43.9	2.5	10.9

#: indiates the sum of the scores of cornea, iris, and conjunctiva.
*: indicate the per cent of area under the score-time curve until 7 days after treatment.

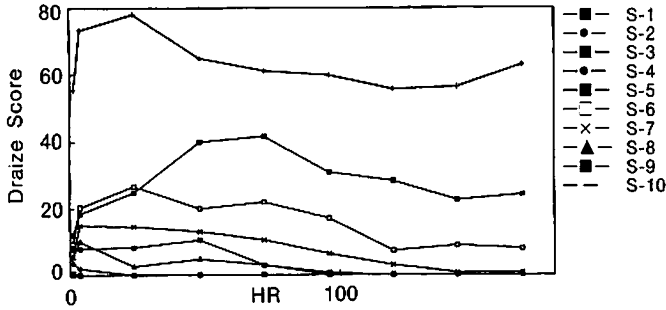
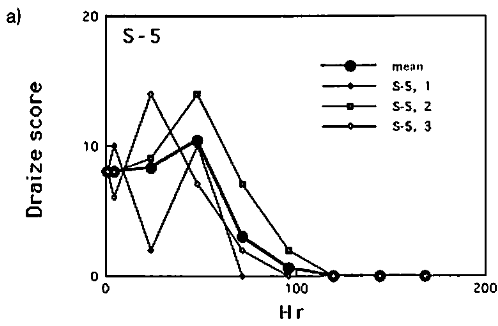
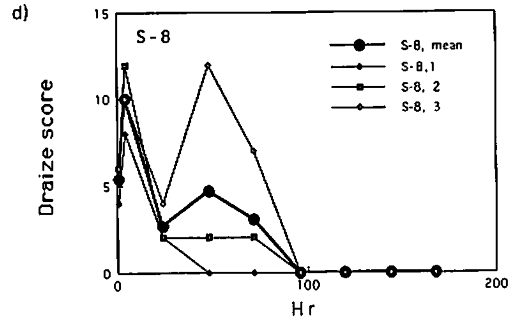


Figure 2. Time-dependent changes of total average scores obtained by the Draize eye irritation test. Ordinate: average of total Draize score obtained from three rabbits. Abscissa: time after application of test substances. Names of the test substances are indicated in Table 3.

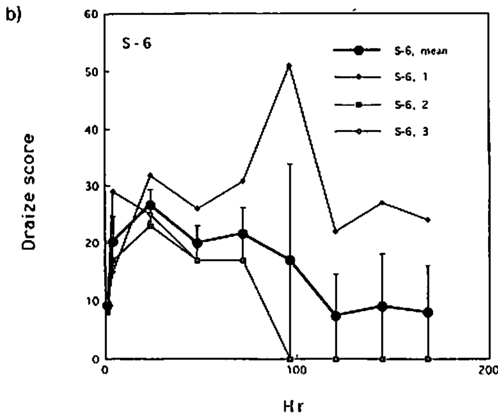
a)



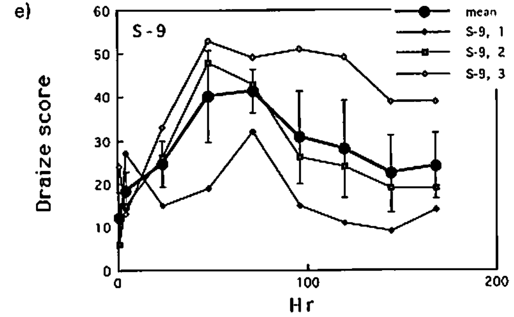
d)



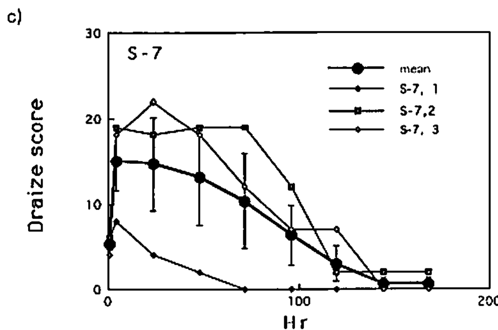
b)



e)



c)



f)

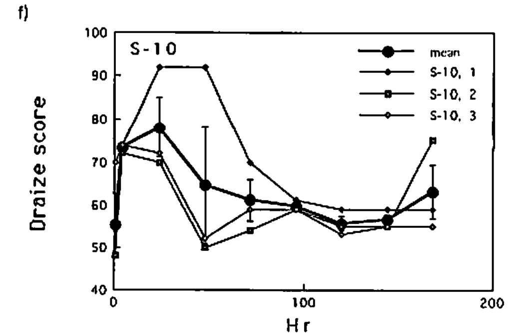


Figure 3. Examples of individual data of Draize eye irritation tests a), b), c), d), e), and f) indicate the results of S-5, S-6, S-7, S-8, S-9, and S-10, respectively. Normal lines indicate results of each rabbit and bold lines indicate the average of those scores. Others are the same as Figure 2.

at 4 or 24 hours after application, and tended to decrease thereafter. However, total scores of Triton X-100 continued to increase until the third day, and total scores of POE laurylether sulfate and PEG monolaurate decreased at 24 hours. These results may indicate that Draize scores taken at specific times do not offer proper evaluation of eye irritation potential. Total scores of SLS was much lower than expectation based on our previous data[15]. The reason for the lower sensitivity to SLS is uncertain. It might be caused by differences of rabbit sensitivity according to individuals or strains. Triton X-100 exhibited slightly lower scores than expected, and HT-glutamate slightly higher scores (unpublished data). Figure 3a)-Figure 3f) indicates the total score of each rabbit and the average of these scores. These results, especially for HT-glutamate, SLS, POE laurylether sulfate and Benzalkonium chloride, indicate that there are large individual differences regarding sensitivity to eye irritants.

2. Results of tests on alternative methods

The results of test on alternative methods are indicated in Tables VI, VII, VIII, and IX.

Sensitivity of the methods

In addition to non-irritants (physiological saline and POE hydrogenated castor oil), there were several substances which did not produce positive results by the RBC or Hb denaturation methods (Tables VI and IX). The RBC test did not produce concentrations of 50% hemolysis for Tween 20, and the Hb denaturation test did not produce denaturation higher than 10% for highest concentrations (1%) of Tween 20, PEG monolaurate, or Triton X-100. RBC and Hb denaturation

Table VI. Results of *in vitro* alternative methods (1)

Sample No.	HET-CAM		CAM-TB		Hemolysis		EYTEX	
S-1	0.35 ± 0.78	(2.24)	0.81 ± 0.76	(0.94)	>20000		13.8 ± 1.2	(0.09)
S-2	1.45 ± 1.16	(0.80)	1.9 ± 1.40	(0.74)	>20000		16.3 ± 3.0	(0.18)
S-3	5.3 ± 3.40	(0.64)	4.03 ± 1.36	(0.34)	>1000		16.6 ± 3.1	(0.18)
S-4	6.95 ± 3.63	(0.52)	5.2 ± 1.92	(0.37)	31.1 ± 39.70	(0.30)	17.1 ± 4.2	(0.24)
S-5	10.3 ± 1.35	(0.13)	10.24 ± 4.51	(0.44)	952.4 ± 187.20	(0.20)	22.9 ± 6.5	(0.28)
S-6	9.6 ± 2.22	(0.23)	10.7 ± 3.27	(0.31)	13.9 ± 4.81	(0.35)	13 ± 4.1	(0.31)
S-7	11.02 ± 0.59	(0.05)	10.09 ± 3.00	(0.30)	16 ± 2.36	(0.15)	38.3 ± 4.3	(0.11)
S-8	8.15 ± 1.59	(0.19)	11.61 ± 4.53	(0.39)	59.9 ± 8.30	(0.14)	23.8 ± 2.6	(0.10)
S-9	10.7 ± 0.91	(0.08)	14.91 ± 6.16	(0.41)	140.1 ± 44.97	(0.32)	15.8 ± 1.6	(0.10)
S-10	13.85 ± 1.61	(0.12)	43.36 ± 10.56	(0.24)	8.2 ± 1.66	(0.20)	36 ± 8.8	(0.24)

These values were mean ± SD with coeffeicient of valiation in parenthesis.
Abbreviations: HET-CAM : macroscopic observation method of hen's chorioallantoic membrane.
CAM-TB : Trypan blue staining method of hen's chorioallantoic membrane.
Data of HET-CAM, CAM-TB, Hemolysis, and EYTEX tests were scores, nmol of trypan blue adsorbed, 50% hemolysis concentration (ug/ml), and scores, respectively.

Table VII. Results of *in vitro* alternative methods (2)

Sample No.	MATREX		SKIN2		CornePack	
S-1	>1000000		>10000		>1000	
S-2	310000 ± 124000	(0.39)	>10000		178.8 ± 58.6	(0.32)
S-3	633 ± 77.7	(0.12)	355 ± 86.4	(0.24)	154.4 ± 84.2	(0.54)
S-4	603 ± 25.2	(0.04)	216 ± 36.5	(0.17)	51.3 ± 20.2	(0.39)
S-5	2620 ± 526	(0.13)	383 ± 87.4	(0.22)	27.9 ± 4.8	(0.17)
S-6	32.5 ± 12.6	(0.38)	29 ± 15.0	(0.50)	1.31 ± 1.09	(0.83)
S-7	167 ± 15.3	(0.09)	47 ± 5.5	(0.11)	0.83 ± 0.18	(0.21)
S-8	557 ± 75.1	(0.13)	168 ± 32.7	(0.19)	4.1 ± 0.71	(0.17)
S-9	408 ± 168	(0.41)	52 ± 6.5	(0.12)	13.5 ± 5.8	(0.43)
S-10	18.9 ± 3.6	(0.19)	3.7 ± 1.2	(0.33)	1.05 ± 0.48	(0.45)

These values were mean ± SD with coeffeicient of valiation in parenthesis.
These data were indicated by concentraion (ug/ml) which inhibited each endopoints by 50%.

Table VIII. Results of *in vitro* alternative methods (3)

Sample No.	SIRC-CV		SIRC-NR		HeLa-MTT		CHL-CV	
S-1	>10000		>10000		>10000		>1000	
S-2	3105 ± 490	(0.16)	2911 ± 1603	(0.55)	4153 ± 1107	(0.26)	1965 ± 551	(0.28)
S-3	766.5 ± 242.6	(0.32)	945.9 ± 229.8	(0.24)	550.6 ± 103.8	(0.18)	202 ± 72.3	(0.35)
S-4	347.8 ± 127.9	(0.37)	427.7 ± 106.7	(0.25)	262.1 ± 45.3	(0.17)	192 ± 68.9	(0.35)
S-5	438.5 ± 57.5	(0.13)	443.9 ± 157.3	(0.35)	372.5 ± 46.4	(0.12)	348 ± 83.5	(0.24)
S-6	139.7 ± 56.1	(0.40)	146.5 ± 34.5	(0.23)	87.4 ± 20.9	(0.23)	52.9 ± 14.5	(0.27)
S-7	167.7 ± 30.1	(0.18)	170.8 ± 25.2	(0.14)	166.3 ± 26.8	(0.16)	188 ± 29.4	(0.15)
S-8	747.4 ± 72.3	(0.10)	675.1 ± 133.9	(0.19)	562.5 ± 148.4	(0.26)	668 ± 133	(0.19)
S-9	38.4 ± 14.2	(0.37)	41.8 ± 16.8	(0.40)	37.0 ± 28.1	(0.75)	35.1 ± 18.1	(0.51)
S-10	19.0 ± 6.5	(0.34)	18.0 ± 6.4	(0.35)	8.6 ± 3.9	(0.45)	20.2 ± 5.8	(0.28)
SLS	162.2 ± 33.9	(0.20)	169.9 ± 25.3	(0.14)	164.6 ± 28.6	(0.17)	178 ± 23.6	(0.13)

These values were mean ± SD with coefleicient of valiation in parenthesis.
Abbreviations: SIRC-CV: crystal violet staining method using SIRC cells, SIRC-NR: neutral red uptake method using SIRC cells, HeLa-MTT: MTT reduction method using HeLa cells, CHL-CV: crystal violet staining method using CHL cells.
These data were indicated by concentraion (ug/ml) which inhibited each endopoints by 50%.
SLS means sodiun lauryl sulfate used as a positive control.

Table IX. Results of *in vitro* alternative methods (4)

Sample No.	Hb denaturation (1% solution of test substances)	
S-1		
S-2	1.59 ± 3.34	(2.10)
S-3		
S-4	5.83 ± 3.86	(0.66)
S-5	27.26 ± 10.49	(0.38)
S-6	29.27 ± 23.94	(0.82)
S-7	42.58 ± 11.76	(0.28)
S-8	31.54 ± 8.21	(0.26)
S-9		
S-10	44.48 ± 8.78	(0.20)

These values were mean ± SD with coefleicient of valiation in parenthesis.
These values were indicated by ratio of denaturated hemoglobin.
Abbreviation: Hb: hemoglobin
Blank column means that denaturations were not observed.

tests are simple and useful methods to determine direct effects of chemicals on cell membranes and proteins, respectively. However, evaluation of eye irritancy by these methods alone can lead to falsely negative results for some compounds.

CornePack had the highest sensitivity among the cultured cell methods showing responses at lower doses. Thus, 50% inhibition doses of test substances for each test method were compared to those of CornePack (Table X). The ratio varied depending on the test substance. However, their average indicated overall sensitivity. From these calculations, SKIN^{2TM} was 2~56 (mean 18) times less sensitive than CornePack. SIRC-CV, SIRC-NR, HeLa-MTT, and CHL-CV were about 50~60 times less sensitive than CornePack. This seemed to be related to the absence of serum in the cell culture medium of CornePack. The other cultured cell methods used culture media containing 10% serum, except for SKIN^{2TM} (2%). Sensitivity of MATREX^{TM} to POE hydrogenated castor oil and to Triton X-100 was lower than that of the other cultured cell methods, but with regard to other substances was about the same.

Table X. Relative sensitivity of several alternative methods to CornePack.

Sample No.	MATREX	SKIN2	CornePack	SIRC-CV	SIRC-NR	HeLa-MTT	CHL-CV
S-1							
S-2	1733.78		1	17.37	16.28	23.23	10.99
S-3	4.10	2.30	1	4.96	6.13	3.57	1.31
S-4	11.75	4.21	1	6.78	8.34	5.11	3.74
S-5	93.91	13.73	1	15.72	15.91	13.35	12.48
S-6	24.81	22.14	1	106.64	111.83	66.72	40.38
S-7	201.20	56.63	1	202.05	205.78	200.36	226.39
S-8	135.85	40.98	1	182.29	164.66	137.20	162.80
S-9	30.22	3.85	1	2.84	3.10	2.74	2.60
S-10	18.00	3.52	1	18.10	17.14	8.19	19.24
mean (n=9)	250.40		1.00	61.86	61.02	51.16	53.32
mean(n=8)	64.98	18.42	1.00	67.42	66.61	54.65	58.62

These values indicated the ratio of EC50 values to those of CornePack for each test substances.

Table XI. Reproducibilities of the test methods and their correlations to Draize scores.

Methods	Reproducibility	Correlation to Draize scores		Discrimination potency			
				Draize score 15		Draize score 20	
	Coefficient of variance	Correlation coefficient	Rank correlation coefficient	False-negative	False-positive	False-negative	False-positive
Chorioallantoic membrane (CAM)							
HET-CAM	0.500 (10)*	0.748 (10)*	0.936 (10)		S-5,8		S-5,7,8
CAM-TB	0.448 (10)**	0.954 (10)**	0.912 (10)		S-5,8	S-6	
Red blood cell							
Hemolysis	0.237 (7)	0.738 (7)***	0.828 (10)	S-9	S-8	S-9	S-7
Hb denaturation	0.321 (7)#	0.661 (7)****	0.529 (10)	S-9	S-5,8	S-9	S-5,7,8
Artificial models of skin corium							
SKIN2	0.241 (8)	0.916 (8)	0.900 (10)		S-7		S-7
MATREX	0.198 (8)	0.725 (8)	0.922 (10)		S-9	S-7	S-9
Normal rabbit corneal epithelial cells							
CornePack	0.394 (9)	0.619 (9)	0.856 (10)		S-8	S-9	S-7,8
Established cell line of rabbit corneal origin (SIRC)							
SIRC - CV	0.256 (10)##	0.894 (9)	0.961 (10)		S-4		S-7
SIRC - NR	0.288 (10)##	0.913 (9)	0.961 (10)				S-7
Established cell line of mammalian origin							
HeLa - MTT	0.280 (10)##	0.848 (9)	0.937 (10)		S-4		
CHL - CV	0.280 (10)##	0.816 (9)	0.903 (10)		S-3,4		
EYTEX	0.186 (10)*	0.481 (10)*	0.324 (10)	S-6,9	S-5,8	S-6,9	S-5,7,8

*, **, ***, ****: Coefficients of variance and correlation coefficients were calculated from the scored values, absorbed trypan blue (nmole/egg), 1/(50% hemolysis concentration), and % denaturation of hemoglobin by 1% solution; respectively. Coefficients of variance and correlation coefficients for others were calculated from 50% cytotoxic concentration and its logarithm, respectively.

Discrimination potencies were estimated by using Draize score 15 or 20 on linear regression lines to discriminaate the test chemicals as positive or negative.

: calculated from the data exceeded 10% denaturation by 1%, 0.125, or 0.01% solution of the test chemicals.

##: calculated from the data including positive control (SLS).

Inter-laboratory reproducibility

Inter-laboratory reproducibility was assessed by comparing the mean coefficient of variation of each method (SD/mean; coefficient of variants: CV) (Table XI). The rank order of each method with regard to inter-laboratory reproducibility is as follows: EYTEX® (mean CV: 0.186, n=10) <MATREX™ (0.198, n=8) <Hemolysis (0.237, n=7) <SKIN2™ (0.241, n=8) <SIRC-CV (0.256, n=10) <HeLa-MTT (0.280, n=10) <CHL-CV (0.280, n=10) <SIRC-NR (0.288, n=10) <Hemoglobin denaturation (0.321, n=7) <CornePack (0.394, n=9) <CAM-TB (0.448, n=10) <HET-CAM (0.500, n=10). The higher variation of the CAM-TB and HET-CAM methods was caused by variability in physiological saline and POE hydrogenated castor oil scores, where very low scores caused the coefficients of variation to increase. On the other hand, data from these substances were not utilized for most of the other methods because numeric data could not be obtained. If these data were exempted from calculation for the CAM-TB and HET-CAM methods as they were for most of the others, the results would be 0.350 and 0.245, respectively. Variation in CornePack was also relatively large. Because the sensitivity of CornePack is higher than that of the other cultured cell methods, extensive dilution might be one of the causes of its relatively large variation. For example, the CV's of HT-glutamate (EC_{50}=1.31 μg/ml), Triton X-100 (13.5 μg/ml), and Benzalkonium chloride (1.05 μg/ml) were 0.832, 0.430, and 0.457, respectively. However, the CV of Tween 20, which has a relatively high EC_{50} (154 μg/ml), was also high (0.545). Thus, another explanation for this variation might be offered. This relatively large variation may be explained in part by the differences in cell growth after seeding the culture dishes arizing from slight differences in the techniques of harvesting cells from preculture bottles. To overcome this difficulty, we only utilized data that had an absorbance higher than 0.500 in controls.

Correlation with Draize scores

As shown in Table XI, the results indicate that correlation coefficients of *in vitro* methods and *in vivo* Draize scores (maximal average Draize total score: MAS) are high for CAM-TB (r=0.954), SKIN^{2TM} (r=0.916), SIRC-CV (r=0.894), SIRC-NR (r=0.913), HeLa-MTT (r=0.848), and CHL-CV (r=0.816), and low for EYTEX^{TM} (r=0.481). We also examined the correlation of other parameters of the Draize test. The results indicate that CAM-TB correlates rather well (r=0.962) with corneal parameters, and that the HET-CAM, Hb denaturation, and CornePack tests correlate well with conjunctiva parameters (r=0.918, 0.930, and 0.919, respectively).

At 24 hours after application of the test substances, total scores correlated well with CAM-TB and SKIN^{2TM}, and corneal scores correlated with CAM-TB. The AUC ratio of the total and corneal score correlated well with CAM-TB and SKIN^{2TM}, and the ratio of total and conjunctiva scores correlated well with CAM-TB, SKIN^{2TM}, SIRC-CV, SIRC-NR, and HeLa-MTT (r> 0.90)[19-27]. However, MAS generally seemed to correlate better than the other parameters. It is interesting to note that there were relatively good linear correlations between Draize scores and the results of *in vitro* experiments, suggesting possible mechanistic links between the *in vivo* and *in vitro* responses.

Spearman's rank correlation coefficients between the results of *in vitro* methods and MAS were higher than 0.9 for all methods except RBC (0.828), Hb (0.520), CornePack (0.856) and EYTEX® (0.324), indicating that the use of several appropriate chemicals as reference standards for *in vitro* alternative methods might provide a useful classification for eye irritancy of test substances. The rank order of the cytotoxicity of Triton X-100 in the cultured cell methods with serum differed greatly from that in methods without serum.

Comparison of *in vitro* results with maximum average scores, 24 hr scores and AUC gave similar but less correlated results, except for the iris for which observation through damaged corea was sometimes difficult.

Compatibility between in vivo and in vitro test results

The irritation potential of ten test chemicals, predicted by using linear regression formulae, was compared with irritation potential predicted by MAS. When we compared the results of eye irritation between the regression line and MAS using 15 or 20 as a discrimination value between negative and positive, certain chemicals turned out to be either false negative or false positive by several methods (Table XI). These discrimination values were set according to the classification of Kay and Calandra[28], or according to the approximate value usually assigned to slightly cornea-damaging substances.

When the discrimination value was set at 15, Triton X-100 was a false negative in the RBC, HB denaturation, and EYTEX® tests, and HT-glutamate was also a false negative in EYTEX®. On the other hand, Lauroyl sarcosinate was a false positive in HET-CAM, CAM-TB, HB denaturation, and EYTEX® tests and POE Lauryether sulfate was also a false positive in HET-CAM, CAM-TB, RBC, HB denaturation, CornePack, and EYTEX® tests. PEG monolaurate was a false positive in SIRC-CV, HeLa-MTT, and CHL-CV tests. Tween 20 was a false positive in CHL-CV.

When the discrimination value was set at 20, Triton X-100 was a false negative in RBC, Hb, CornePack, and EYTEX^{TM}. SLS was a false positive in HET-CAM, RBC, Hb, SKIN^{2TM}, CornePack, SIRC-CV, SIRC-NR and EYTEX® tests.

The false negative results for Triton X-100 may be attributed to the fact that its protein denaturation activity is weak. The false positive results for SLS are possibly related to its unexpectedly low Draize score obtained *in vivo*. Cultured cell methods using serum did

not show any false negative results.

Correlation between in vitro alternative methods

As mentioned above, false negative and false positive results were observed for most of the test methods. This implies that any decision based on a single method may be flawed. As there are many *in vitro* methods based on relatively independent mechanisms, a combination of these methods decreases the chance of false negative results. Thus, we correlated the results of *in vitro* methods among themselves using the data of six substances for which every test method produced data valid for comparison.

In spite of the differences between cell types and endpoints, data obtained by cell culture methods using serum containing culture media (SIRC-CV, SIRC-NR, HeLa-MTT, CHL-CV) correlated very well ($r=0.92–0.99$). MATREX™ also correlated well with SKIN2™, SIRC-CV, SIRC-NR, and HeLa-MTT ($r>0.94$). The reciprocal value of 50% hemolysis concentration also correlated well with MATREX™, SKIN2™, SIRC-CV, SIRC-NR, and HeLa-MTT ($r>0.93$). As was expected, CornePack did not correlate well with other cultured cell methods ($0.51<r<0.73$). EYTEX® did not correlate with the test methods (mostly $r<0.5$) except for Hb ($r=0.757$), which has endpoint characteristics similar to EYTEX®. Correlation of HET-CAM and CAM-TB with other methods was mostly in the range of 0.5~0.9.

Rank correlation coefficients among cultured cell methods were higher than 0.98. Correlation coefficients of MATREX™ with SKIN2™, CornePack, SIRC-CV, and SIRC-NR were also higher than 0.9. RBC had a good correlation with MATREX™, SKIN2™, and CornePack. Correlation of EYTEX® with other test methods was not good except for Hb ($r=0.701$).

Our results are in line with the results of validation studies done by CTFA[29], which indicated that the results of the HET-CAM method and cultured cell methods using SIRC cells correlated well with those of the *in vivo* Draize test. However, our results are different from those of SDA[30], which reported that the HET-CAM and EYTEX® methods correlated well. The difference between the test substances might be one reason for this discrepancy.

CONCLUSION

It is necessary that alternative methods for the prediction of eye irritancy be based on a scientifically valid mechanism. Cytotoxicity tests may be one candidate to be an alternative to the eye-irritation test with respect to this point, because they afford information about the overall effects of test substances on many basic biochemical mechanisms of cells, which may correspond with the direct effects on ocular cells in *in vivo*.

Cultured cell methods using serum showed relatively good reproducibility and correlation with Draize scores. Among the cells used, SIRC cells were the only established cell line which derived from rabbit corneal epithelial cells. However, Kojima et al[26] indicated that difference in cell type does not seem to cause any significant differences in results compared to the Draize test, as long as the test substances are limited to surfactants.

As for the endpoints of cytotoxicity, crystal violet staining and neutral red uptake methods were compared using SIRC cells. Both methods yielded similar EC_{50} values when the test substances were surfactants.

Although the chemicals tested constitute only one kind of cosmetic ingredient, false negative and/or false positive results were obtained in most of the *in vitro* methods. Thus, batteries of test methods, composed of several different types of *in vitro* methods, seem to be the only valid replacement for *in vivo* testing. The cultured cell methods using serum and HET-CAM seem to be useful as cores of the battery system for the preliminary

evaluation of the eye irritation potency of cosmetic ingredients because of their high correlation with Draize scores. Other methods showing relatively lower correlation with the Draize eye irritation test also may be necessary to cover specific mechanisms of eye irritation.

Further validation studies using a wider range of chemicals used as cosmetic ingredients are required. We are now organizing the next step of the validation program using other types of chemicals.

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