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First Phase Inter-Laboratory Validation of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients: (3) Evaluation of Hemolysis Test

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SUMMARY

A hemolysis test using sheep red blood cell (RBC) as an alternative method to Draize rabbit eye irritation test (Draize test) was evaluated by nine laboratories using 9 surfactants and physiological saline as test substances. The test procedure was controlled under a common standard operating procedure (SOP) among laboratories. The concentrations of test substances that showed 50% hemolysis (HC50) were determined, and was compared *in vivo* Draize test scores. Inter-laboratory coefficients of variation among the nine laboratories were calculated for each test substance and the average of those was 23.7%. In this study, only one test substance, Tween20, had relatively large variation of HC50 values among laboratories. This seemed to be because Tween20 results were affected more sensitively by the slight changes in experimental conditions than the other test substances in this study, because the reproducibility of Tween 20 results was markedly improved in a supplemental test conducted after a detailed reviw of the SOP. Rank order correlation coefficients between hemolysing potencies and Draize scores were relatively good. When 1/HC50 was compared to maximal average Draize total score (MAS), the correlation coefficient was 0.738. From these results and its ability to measure direct effects of chemicals on cell membrane, we considered that the hemolysis test is a promising alternative method to the Draize test. Further

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validation of this method using a wide range of cosmetic ingredients is under way.

INTRODUCTION

The hemolysis test is a method of evaluating membrane injury using red blood cells (RBC) as a model biomembrane and measuring the amount of hemoglobin which leaks from RBC. In the case of membrane injury caused by surfactants, the sites of action of surfactants are lipids and/or proteins in the cell membrane. Hemolysis is considered to be caused by adsorbing of surfactants to RBC membrane and followed by elution of lipids and conformational changes of membran proteins. Cationic surfactants show the most potent hemolytic activity followed by anionic surfactants. Hemolytic activity of nonionic surfactants is considered to be comparatively weak[6–8,10–11,13–15]. The hemolysis test has been studied as an alternative method to Draize eye irritation testing (Draize test)[1] and seemed to be a good candidate for further validation. Thus, we conducted an interlaboratory validation of the hemolysis test as an alternative method to the Draize test under the Ministry of Health and Welfare (MHW) project entitled "Studies on the test methods to evaluate the safety of new ingredients of cosmetics". There are several modified methods for the hemolysis test. Among these methods, there is a method adding evaluation of denaturation of leaked hemoglobin[9]. We chose to evaluate a simple hemolysis method in which denaturation of hemoglobin was not considered.

MATERIALS AND METHODS

Test substances

The names of the 10 test substances used as test materials are listed in Table I. They include one cationic surfactant, 4 anionic surfactants, 4 nonionic surfactants and physiological saline[3]. They were of Japanese standard of cosmetic ingredients[4–5], and supplied from the Japan Cosmetic Industry Assoc. (JCIA) to the National Institute of Health Science (NIHS). Coded substances were supplied from NIHS to each laboratories to enable us to get objective information about the methods.

Test method

1) Preparation of RBC suspension

RBC suspension was prepared by centrifuging (approximately 1500G) sterile and preserved blood of sheep, which is commercially available, at 4°C for 10 min. The pellets were washed with isotonic saline two times and with isotonic phosphate buffer (PBS pH 7.4) once by resuspension and centrifugation. RBC fractions, thus obtained, were suspended in PBS at 400×10^6 RBC/ml. The RBC suspensions were kept at 4°C until use.

Table I. List of test substances

Sample number	Name	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	Polyoxyethyleneglycol Monolaurate (10 E.O.)	PEG monolaurate	monionic
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

2) Test procedure

The study was carried out according to the methods used by Kondo and Tomizawa[6-8], and by Pape et al[9]. Each test substance was dissolved into five different concentrations with PBS. One ml of test substance solution was added into the same volume of RBC suspension in a polystyrene centrifuge tube. The mixture was incubated at 32°C for 30 min. Distilled water or PBS was used as complete hemolysis control or solvent blank, respectively. After 30 min. incubation, the mixture was centrifuged at 1500G for 10 min. at 4°C. The amount of hemoglobin in the supernatant was measured spectrophotometrically at 540 nm and 575 or 577 nm. The measurement at 575 or 577 nm was conducted to confirm denaturation of hemoglobin in the supernatant. If an absorbance difference between 540 nm and 575 nm was detected, HD occurred. In this case, the study was performed within a concentration range that did not produce HD. Percent hemolysis was calculated from absorbance of test samples at 540 nm and those of the completely hemolyzed control. HC 50 (the concentration at which 50% hemolysis of RBC suspension was caused) was calculated from doseresponse curves showing the relationship between percent hemolysis (%) and test substance concentrations. The test procedure was controlled under the same standard operating procedure (SOP) among laboratories.

RESULTS

1) Comparison of HC50 values obtained by different laboratories;

Nine laboratories participated in this validation study of the RBC hemolysis test. The results of the evaluation obtained by each laboratory are shown in Table II and III. Intra-laboratory and inter-laboratory reproducibility of data was satisfactory except for Tween 20. Coefficients of variation among laboratories were lower than 34% for the other seven substances for which an HC50 was obtained. Concerning Tween 20, there were relatively large variations of results even with laboratories. In order to investigate the cause of such variability, a supplementary test was performd at several laboratories in which effects of newly supplied Tween 20 from the stock sample were compared with those of already supplied Tween 20. The results of the supplemental test are shown in Table IV and V. Consequently, the variations were markedly improved.

2) Correlation between HC50 values and the results of in vivo Draize test;

The correlation coefficient and the Spearman's rank correlation coefficient between the results of the hemolysis test (the supplemental data were included) and the *in vivo* Draize test data of Ohno et al[2] (Table VI) are shown in Table VII. The correlation graphs are shown in Fig. 1 and 2. Higher correlation coefficients were not obtained as a whole in the comparison between HC50 values and the results of Draize test. The correlation between HC50 values and the scores evaluating conjunctiva were relatively high. However, those for iris was low. Concerning the *in vivo* Draize data, they correlated well with 1/HC50 as a whole, but showed lower correlation with log HC50. Rank order of hemolysis potency correlated relatively well with those of Draize scores. The test substance that exhibited lower concordance with the rank of *in vivo* Draize data was Triton X-100.

3) Compatibility of classification of eye irritation potencial with that by in vivo test;

Because the correlation coefficient was relatively high in the case of 1/HC50, predictability of hemolysis tests for irritation potency was assessed by using regression lines comparing 1/HC50 values and the maximum average Draize total scores (MAS) for seven test substances (Fig. 1 and Fig. 2) for which HC50 values could be estimated. The cut-off point was set at MAS 15. Eye irritation of chemicals with scores greater than this cut-off point were

Table II. Results of HC50 values of hemolysis method on each laboratories

Sample No.		HC50(μg/mℓ) of each laboratories								
		A	B	C	D	E	F	G	H	I
S-1		20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<
		20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<
		20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<
	AV.	20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<
	SD.	0	0	0	0	0	0	0	0	0
	CV.									
S-2		20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<
		20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<
		20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<
	AV.	20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<	20000<
	SD.	0	0	0	0	0	0	0	0	0
	CV.									
S-3		10800	1800	977.0	285.0	5000	20000<	5416	2000	187.0
		10000	1200	540.0	400.0	4800	20000<	5238	5800	197.0
		11000	800.0	10500	500.0	4100	20000<	7152	4200	180.0
	AV.	10600	1267	4006	395.0	4500	20000<	5935	4000	188.0
		(20000<)		(20000<)	(20000<)		(20000<)			(20000<)
	SD.	529.2	450.2	5629	107.6	500	0	1057	1908	8.54
	CV	0.017	0.355	1.405	0.270	0.110		0.178	0.477	0.045
S-4		118.0	132.0	151.5	88.0	220.0	111.0	107.7	152.0	114.5
		115.0	133.0	76.5	98.0	232.5	111.5	117.4	175.0	106.0
		120.0	128.0	113.0	110.0	220.0	111.0	110.4	165.0	101.5
	AV	117.7	131.0	113.7	98.7	224.3	111.2	111.8	164.0	107.3
	SD.	2.05	2.16	30.6	8.99	6.13	0.24	4.09	9.42	5.39
	CV.	0.017	0.016	0.269	0.091	0.027	0.002	0.037	0.057	0.050
S-5		1010	1170	364.0	890.0	900.0	1040	1034	850.0	1140
		1000	1125	565.0	920.0	870.0	1020	1095	980.0	1100
		1050	1115	600.0	950.0	920.0	900.0	1024	950.0	1100
	AV.	1020	1148	509.7	920.0	896.7	986.7	1051	926.7	1110
	SD.	26.5	22.5	127.4	30.0	25.2	75.7	38.2	68.1	23.1
	CV.	0.026	0.020	0.250	0.033	0.028	0.077	0.036	0.073	0.021
S-6		10.6	18.0	24.5	6.6	9.1	22.0	14.3	10.5	9.0
		11.2	19.6	10.2	11.2	9.5	22.0	16.7	22.0	9.0
		11.0	21.0	26.5	7.4	8.5	14.0	12.7	11.5	9.0
	AV.	10.9	18.9	20.4	8.40	9.0	19.3	14.6	14.7	9.0
	SD.	0.31	2.58	8.89	2.46	0.50	4.6	2.01	6.37	0
	CV.	0.028	0.140	0.440	0.290	0.056	0.240	0.140	0.430	
S-7		19.4	16.0	20.0	14.0	16.5	15.0	17.1	25.0	13.0
		18.8	15.5	2.8	14.8	16.8	13.5	16.9	17.5	12.7
		18.5	16.8	17.5	16.8	16.6	14.0	16.4	16.5	12.5
	AV.	18.9	16.1	13.4	15.2	16.6	14.2	16.8	19.7	12.7
	SD.	0.46	0.66	9.32	1.44	0.15	0.76	0.36	4.65	0.25
	CV.	0.024	0.041	0.670	0.090	0.009	0.054	0.021	0.240	0.020
S-8		49.0	60.6	81.0	48.5	55.0	68.5	53.1	86.0	64.5
		49.0	63.0	73.0	58.2	47.0	55.5	62.8	67.0	63.5
		60.0	54.0	70.0	52.0	47.0	61.0	51.4	56.0	62.5
	AV.	52.7	59.2	74.7	52.9	49.7	61.0	55.8	69.7	63.5
	SD.	6.35	4.66	5.69	4.91	4.62	5.50	6.15	15.2	1.0
	CV.	0.120	0.079	0.076	0.093	0.093	0.090	0.110	0.220	0.016
S-9		104.0	134.5	95.0	89.0	260.0	136.0	138.2	135.0	127.0
		118.0	156.5	155.0	120.0	252.0	132.0	128.5	135.0	128.0
		119.0	136.0	112.0	120.0	260.0	132.0	128.6	135.0	114.0
	AV.	113.7	135.7	120.7	109.7	257.3	133.7	131.8	135.0	123.0
	SD.	8.39	0.93	30.9	17.9	4.26	2.08	5.57	0	7.81
	CV.	0.074	0.007	0.258	0.163	0.018	0.016	0.042		0.063
S-10		6.2	8.0	9.1	6.8	10.3	7.7	7.3	6.3	9.7
		5.9	8.1	11.0	7.4	10.5	7.5	8.2	5.9	9.4
		7.8	9.1	11.5	7.9	10.3	7.2	7.2	5.0	9.4
	AV.	6.60	8.40	10.5	7.40	10.4	7.5	7.6	5.7	9.5
	SD.	1.02	0.61	1.27	0.55	0.12	0.26	0.55	0.67	0.17
	CV.	0.150	0.083	0.120	0.074	0.012	0.035	0.072	0.12	0.018

AV.: Average of HC50 values
SD.: Standard deviation
CV.: Coefficient of variation

* The data in the parenthesis are the results of the supplemental test.

Table III. Summary statistics of HC50 values of hemolysis test

Sample No.	N	Average of HC50 (μg/mℓ)	S.D.	Coefficient of Variation	Rank of *in vivo* Data(Ohno et al)*
S－1	9	20000<			1－2(weak)
S－2	9	20000<			1－2
S－3	8	3861.4(1000<)	3430.9	0.889	3
S－4	9	131.1	39.7	0.302	4
S－5	9	952.4	187.2	0.197	6
S－6	9	13.9	4.8	0.345	8
S－7	9	16.0	2.4	0.150	7
S－8	9	59.9	8.3	0.139	5
S－9	9	140.1	45.0	0.321	9
S－10	9	8.2	1.7	0.207	10(sever)

The data in the parenthesis are the results of the supplemental test.

* Results of using the maximum average scores (MAS)

Table IV. Results (HC50: μg/ml) of supplemental test; (stability of Tween 20)

Sample	Laboratories				
	A	C	D	F	I
Tween20 (stored)	20000<	20000<	20000<	20000<	20000<
Tween20 (newly supplied)	20000<	20000<	20000<	20000<	20000<

Table V. Results (HC50: μg/ml) of supplemental test; (effect of incubation temperature and time)

Sample	32℃		37℃	
	30min.	60min.	30min.	60min.
Tween20 (stored)	20000<	–	1000>	1000>
Tween20 (newly supplied)	20000<	5800	1000>	1000>
TritonX-100 (as a control)	113.7	120	–	–

Table VI. Results of Draize rabbit eye irritation test on 10% test substances. (Ohno et al[2])

Sample number	Maximum average score				24hrs score				Area ratio under curve* %			
	Total	Cornea	Iris	Conjuctiva	Total	Cornea	Iris	Conjuctiva	Total	Cornea	Iris	Conjuctiva
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(24-72)	1.7(72-168)	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	10.0	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(4,48)	24.7	15.0	1.7	8.3	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 7days after treatment.

** Sample names show in Table I.

*** These values in parenthesis are the time (hour) when the scores became maximum.

Table VII. Correlation coefficients and Sparman's rank correlation between HC50 values and Draize scores

		Correlation Coefficients			
		HC50	1/HC50	log.HC50	Rank correlation
Maximal Average Draize scores (MAS)	Total	-0.318	0.738	-0.527	0.818
	Corneal	-0.224	0.730	-0.452	0.693
	Iris	-0.063	-0.134	0.238	0.358
	Conjuctiva	-0.780	0.720	-0.882	0.976
Scores at 24 hrs. after	Total	-0.303	0.849	-0.603	0.827
	Corneal	-0.272	0.833	-0.568	0.736
	Iris	-0.063	-0.331	0.238	0.336
	Conjuctiva	-0.414	0.796	-0.722	0.836
Area Under curve(AUC);%	Total	-0.314	0.756	-0.508	0.809
	Corneal	-0.311	0.753	-0.527	0.723
	Iris	-0.301	0.483	-0.343	0.639
	Conjuctiva	0.317	0.729	-0.527	0.908

Table VIII. Reproducibility of the HC50 to the MAS, in case that the MAS was discriminated at score 15.

Classification		*in vivo* results	*in vitro* results	
			1/HC50	log.HC50
False	Positive	–	1 (S-8)	2 (S-4, S-8)
	Negative	–	1 (S-9)	0
True	Positive	4	3	4
	Negative	6	5	4

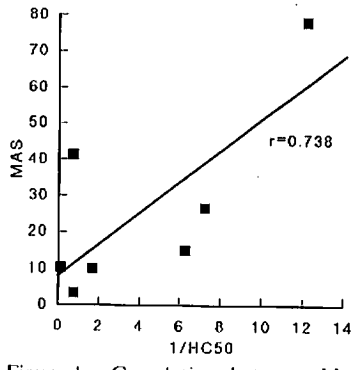

Figure 1. Correlation between Maximal average Draize scores (MAS) and 1/HC50

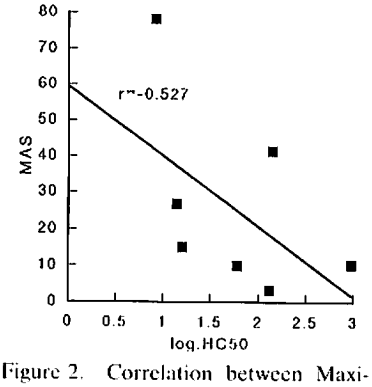

Figure 2. Correlation between Maximal average Draize scores (MAS) and log. HC50

regarded as positive and those with lower scores were regarded as negatives. When we examined the results for ten substances according to this formula, there was one false positive substance (P.O.E. laurylether sulfate) and one false negative substance (Triton X-100) (Table VIII). The correspondence rate between the *in vivo* and *in vitro* data was 80%.

DISCUSSION

The hemolysis test is simple in test operation, and does not need special techniques and expensive equipment. This method has a merit of being rapid. In this study, we examined whether the present test method is useful as an alternative to the Draize test. Reproducibility of the test seemed to be good for all compounds except for Tween20. The cause of this variation in Tween20 might be due to variation in procedure for mixing test substance with the RBC suspensions, and minute differences in incubation temperature and time. It might also due to the stability of Tween20. Thus, we reconfirmed in detail the experimental steps in the SOP, and carried out a supplemental test at several laboratories using the stored Tween20 in each laboratory and a newly supplied one from NIHS. We found that the reproducibility of data obtained for Tween20 was markedly improved, there were no differences in the effects between the two lots of Tween20 and hemolysis by Tween20 were affected more sensitively by the slight changes in incubation time and temperature than the other test substances in this study. Except for Tween20, the intra-laboratory coefficient of variations for test substances were 0–44%, and the inter-laboratory coefficient of variation was 13–34% (average 23.7%). From these results, the reproducibility of the hemolysis test is considered to be satisfactory when performed strictly in compliance with the SOP.

Since there were three substances in which an HC50 could not be obtained in accordance with their minimal eye irritating properties, the correlation coefficient with Draize scores was estimated for seven test substances. The correlation coefficient for HC50 vs. *in vivo* responses was not as good as for cytotoxicity tests in cultured cells[2]. However, when we converted the data to 1/HC50, it became

better. On the other hand, conversion to log HC50 did not bring satisfactory correlation with Draize scores The reason why reciprocal value of HC50 correlates better than logHC50 and whether or not this applies also to the other types of compounds are uncertain. We will examine this issue again after getting data for many more substances. The correlation between HC50 values and the scores evaluating conjunctiva were reletively satisfactory. However, the correlation between HC50 values and the scores in the iris evaluation was lower, probably due to the fact that there are only three substances which showed changes in the iris and that the iris responses are evaluated only into three grades (0, 1, 2). The Spearman's rank correlation between the HC50 values and *in vivo* data seemed to be satisfactory except for the iris scores. *In vitro* results for Triton X-100 seemed to be lower than those from the *in vivo* method. The characteristics of the *in vivo* data for Triton X-100 was that the peak of irritation was delayed as compared to the other surfactants[2]. On the other hand, the hemolysis test detects membrane injury occurring within a relatively short period after the application of test substances and reflect usually direct physico-chemical interaction between biomembrane and the test substances. Thus, this test may not be suitable for the evaluation of the test substances with which the *in vivo* reactions are promoted step by step, while the test is suitable for evaluating the test substances with which the reactions occur within relatively short period.

In the evaluation of cosmetic ingredients, by Draize test, it is critical to know whether the chemicals may cause corneal damage or not. The draize score which corresponds to the occurrence of irritation in cornea is around 15, which is categorized into "mild irritant", according to the classification of Kay and Calandra[20]. Thus we examined the compatibility of classification of eye irritation potencial by hemolysis test with that by *in vivo* test using cut-off point 15 by MAS (Table VI). Abscissa values corresponding to Draize score 15 on the regression line of MAS on 1/HC50s were 2.66×10^{-2} $\mu g^{-1}/ml^{-1}$ (HC50=37.5 μg/ml) respectively.

The correspondence of classification by using regression line of MAS on 1/HC50s and these cut-off points to be those by MAS was 80%. The correspondence seemed to be satisfactory. A false negative result was obtained only for Triton X-100. Triton X-100 is a nonionic surfactant, and is known to have minimal protein denaturing action.

We showed that its hemoglobin denaturation effects was also low (Itagaki et al[19]). These results suggest that cell membrane protein denaturation plays one of the major role in hemolysis and that significant eye irritation may be caused without protein denaturation. From the present results and discussion, we suggested that the hemolysis test can be useful as an alternative method to Draize test. However, in order to reach such a conclusion, it is necessary to carry out further large scale validation tests.

Tentatively, we propose the criteria for classification of eye irritation potential by the hemolysis test. That is when HC50 values are more than 1000 g/ml the chemicals are evaluated as negative and when HC50 values are less than 20 g/ml, the chemicals are evaluated as positive. However, further studies are also needed for the establishment of the grading system.

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REFERENCES

1) Draize, J.H., Woodward, G. and Calvery, H.O. (1944) Methods for the study of irritation and toxicity of substances applied to skin and mucousa membrane, *J. Pharmacol. Exp. Ther.*, **82**, 377–390.

2) Ohno, Y., Kaneko, Kobayashi, T., Inoue, T., Kuroiwa, Y., Yoshida, T., Momma, J., Hayashi, M., Akiyama, J., Atsumi, T., Chiba, K., Endo, T., Fujii, A., Kakishima, H., Kojima H., Masamoto, K., Masu-

da, M., Matsukawa, S., Ohkoshi, K. Okada, J., Sakamoto, K., Takano, K. and Takanaka, A. (1994) First phase validation of the *in vitro* eye irritation tests for Cosmetic ingredients, *In Vitro Toxicology* 7, 89–94.
3) *The Pharmacopoeia of Japan, Twelfth Edition* (1992) Yakuji Nippo, Ltd., Tokyo.
4) *The Japanese Standards of Cosmetic Ingredients Second Edition* (1985) Yakuji Nippo, Ltd., Tokyo.
5) *The Comprehensive Licensing Standards of Cosmetics by Category, Part I* (1986) Yakuji Nippo, Ltd., Tokyo.
6) Kondo, T. and Tomizawa, M. (1968) Hemolysis by nonionic surface-active agents, *J. Pharm. Sci.*, **57**, 1246–1248.
7) Kondo, T. and Tomizawa, M. (1968) Release of lipids from red cell membrane by surface-active agents, *Chem. Pham. Bull.*, **16**, 738–740.
8) Kondo, T. and Tomizawa, M. (1970) Effect of ionic head of cationic surface-active agents on their hemolysis activity, *Chem. Pham. Bull.*, **18**, 2150–2163.
9) Pape, W., Pfannenbecker, U. and Hoppe, U. (1987) Validation of red blood cell test system as *in vitro* assay for the rapid screening of irritation potential of surfactants, *Molecular Toxicology*, **1**, 525–536.
10) Ossipov, N.N., Zaslavsky, B.Y., and Rogozhin, S.V. (1978) Action of surface-active substances on biological membranes, *Colloid & Polymer Sci.*, **256**, 1105–1109.
11) Bettley, F.R. (1968) The toxicity of soap and detergents, *Br. J. Derm.*, **80**, 635–642.
12) Isomma, B., Lilius, H. are West, A. (1992) Evaluation of cytotoxicity of the first twenty MEIC chemicals by using haemolysis of human erythrocytes as an endpoint, *ATLA*, **20**, 226–229.
13) Okamoto, Y., Kanzaki, N and Tanaka, N., (1990) Studies of an *in vitro* alternative method to the Draize rabbit eye irritation test, *J. Soc. Cosmet. Chem. Japan*, **23**, 272–279.
14) Anson, M.L. (1945) Protein denaturation and properties of protein groups, *Adv. Protein Chem.*, **2**, 361–386.
15) Tanford, C. (1976) Characterization of membrane proteins in detergent solutions, Biochemica et *Biophysica Acta*, **457**, 133–170.
16) Borenfreund, E. and Puerner, J.A. (1985) Toxicity determined *in vitro* by morphological alternations neutral red absorption, *Toxicol. Letters.*, **24**, 119–124.
17) Kemp, R.B., Merdith, R.W.J., Gamble, S. and Frost, M. (1983) A rapid cell culture technique for assessing the toxicity of detergent-based products *in vitro* as a possible screen for eye irritancy *in vivo*, *Cytobios*, **36**, 153–159.
18) North-root, H., Yacovich, F. and Demetrulias, J. (1982) Evaluation of an *in vitro* cell toxicity test using rabbit corneal cell to the eye irritation potential of surfactants, *Toxicol. Letters.*, **14**, 207–212.
19) Itagaki, H., Hayashi, T., Kakishima, H., Ogawa, T., Kotani, M., Matsukawa, K., Masuda, K., Kojima, H., Chiba, K., Makino, I., Sakamoto, K., Takino, Y., Kanari, M., Kaneko, T., Hirose, A., and Takanaka, A. (1994) First phase inter-laboratory validation of the *in vitro* eye irritation tests for cosmetic ingredients, (4) Evaluation of Hemoglobin Denaturation (HD) test, *AATEX*, **3**, 154–161.
20) Kay, J.H. and Calandra, I.C., (1962) Interpretation of Eye irritation tests, *J. Soc. Cosm. Chem.*, **13**, 281–289.