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First Phase Inter-Laboratory Validation of the *In Vitro* Eye Irritation Tests for Cosmetic Ingredients: (10) Evaluation of the EYTEX® Method

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SUMMARY

The EYTEX® method was evaluated in five laboratories as an alternative method to predict the eye irritation of cosmetic ingredients. Nine surfactants and Isotonic Sodium Chloride Solution were evaluated as coded samples. Two of the nine surfactant samples studied, Sodium Hydrogenated Tallow-L-glutamate and Polyoxyethylene Octylphenylether (10 E.O.), were found to suppress or inhibit the EYTEX® reaction system so that the test results obtained with these samples were invalid and had a low degree of correlation with the *in vivo* study results. However, the test results for the remaining 8 samples were valid, showing a small degree of interlaboratory variation (the coefficient of variation: 25% or less). A high correlation coefficient was obtained between the scores for the eight samples obtained by the EYTEX® method and those from the *in vivo* test method. The correlation coefficient was high in particular in comparison to the evaluation of conjunctival reactions, 0.884 for the maximum score, 0.955 for the 24h score and 0.767 for the area under the curve (AUC). The corresponding values for maximal average total Draize score (MAS) were 0.710, 0.703, and 0.657, respectively. The irritation ranking for the eight samples, excluding for the above-mentioned two samples, as determined by the EYTEX® method corresponded with those obtained by the *in vivo* test.

Even though two substances were not compatible with The EYTEX® method, we concluded that EYTEX® is a promising alternative method to the Draize eye irritation test (Draize test) based on the results obtained for the remaining 8 samples and the relatively simple assay procedure. Further validation of this method using a wider range of cosmetic ingredients is under way.

INTRODUCTION

The EYTEX® method, developed by *In Vitro* International, in the United States, is an *in vitro* test method used to predict the Draize eye irritation. The method utilizes the changes in the hydration state and structure of a regularly arranged, high molecular weight protain matrix as an indicator of the irritation potential of the test substance. The main ingredient of the reagent used in this method is oligomeric protein (mw; ca 300,000) consisting of 12 subunits. Oligomers themselves

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fibrously bind to one another to form the high molecular weight protain matrix. This protain matrix contains in a buffer solution low molecular weight proteins, peptides, amino acids, amino glycanes and mucopolysaccharides in addition to oligomeric protein. It also contains an accelerator and a stabilizer to enhance the stability of the matrix itself and its reactivity wih many chemical substances including acids, bases, salts, solvents, surfactants, lubricants, antiseptics, emulsions, dyes, amines and amides.

Using the EYTEX® method, many chemical substances and drugs have so far been evaluated for their irritation potential. In the validation tests of a wide variety of substances having different toxicological mechanisms and degree of toxicity, good correlations have been reported between the results obtained by this method and those obtained by the *in vivo* Draize test[1-9].

We conducted a first phase inter-laboratory evaluation of the EYTEX® method using nine coded surfactants and physiological saline as a negative control by five independent laboratories under the same Standard Operating Procedure (SOP). The results are presented and discussed in this report, which is a part of the MHW project entitled "Studies on the Test Methods to Evaluated the Safety of New Ingredients of Cosmetics."

MATERIALS AND METHODS

Test substances

The names of the 10 test substances used as the test materials are listed in Table 1. They consisted of one cationic surfactant, 4 anionic surfactants, 4 nonionic surfactants and one Isotonic Sodium Chloride Solution[10]. They were listed on the Japanese standards of cosmetic ingredients[11,12], and were supplied by the Japan Cosmetic Industry Association (JCIA) to the national Institute of Health Science (NIHS). The coded substances were distributed to each laboratory to enable us to get objective information about the methods and intra-/inter-laboratory variations.

Each surfactant was dissolved or suspended in distilled water at 10% (W/V) concentration and used for both the *in vivo* and *in vitro* tests. (All other reagents were obtained commercially and were of the highest grade available.)

The EYTEX® kits were supplied to each

Table 1. List of test substances

Sample number	Name	Abbreviation	Classification
S-1	Isotonic Sodium Chloride Solution	Physiological saline	-
S-2	Polyoxyethylene Hydrogenated Caster Oil(60 E.O.)	POE hydrogenated castor oil	Nonionic
S-3	Polyoxyethylene Sorbitan Monolaurate (20 E.O.)	Tween 20	Nonionic
S-4	Polyethyleneglycol Monolaurate (10E.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 (2E.O.; 27%solution)	POE laurylether sulfate	Anionic
S-9	Polyoxyethylene Octylphenylether (10E.O.)	Triton X-100	Nonionic
S-10	Benzalkonium	Benzalkonium chloride	Cationic

laboratory by InVitro International located in the United States.

The validation study was carried out in accordance with the "EYTEX® SYSTEM MANUAL", protocol included in each test kit.

Procedures

1) Measure 30 ml of EYTEX Water (W) into the EYTEX Reagent (R). Gently swirl the rehydrated EYTEX Reagent. Filter and check the pH of the (R) using a pH meter.
2) Set up the cuvettes for the EYTEX protocol.
3) Measure 25 ml of (R) and Diluent (D), then add 500 μl of Activator (A) into both of the 25 ml of (R) and (D). Activator, EYTEX Reagent and Diluent should be at 25°C for optimal results.
4) Check the pH of the activated (R) using a pH meter prior to use.
5) Add 1 ml of appropriately activated (R) to the following cuvettes; CR0, CR1, CR2, QC1, QC2, TS10, TS20, TS30, TS50 and TS100.
6) Add 1 ml of activated (D) to the following cuvettes; B0, BQC1, BQC2, B10, B20, B30, B50 and B100.
7) Add Calibrators (CR0–CR3), Quality Controls and samples into the Bullets membrane, insert into the appropriate cuvette and cap immediately.
8) Incubate for 24 hours (±30 minutes) at 25°C. Temperature must be carefully maintained.
9) Turn Colorimeter on/off switch to the ON position 15 minutes before use, ensure the filter wave length is set at 400 nm.
10) After incubation, agitate the rack gently back and forth for 15 seconds, remove caps and withdraw each Bullet membrane after dipping several times in its respective solution, then discard.
11) Set the colorimeter to zero with B0. Read and record CR0. Set the instrument to zero with the CR0 cuvettes. Then read and record each respective CR, QC and sample cuvette. Reset the instrument to Zero on the B0 cuvette, read and record each respective blank cuvette.
12) The DAQC-ES program will allow the entry of data, the Optical Density readings from the Colorimeter. The program will then determine assay performance, using the Calibrators and Quality Controls. If an assay is qualified, then the test samples EDE (EYTEX DRAIZE EQUIVALENT) scores for the dose response will be calculated.

Representatives from each laboratory received a brief, one day training course about the EYTEX® protocol from the supplier.

RESULTS

Table 2 shows the results obtained using the EYTEX® method at each of the five testing facilities, as well as the mean value, the standard deviation, the coefficient of variation of the results, and also the irritation ranking specified in Table 3. As a result of these assays, Isotonic Sodium Chloride Solution and Sodium Hydrogenated Tallow-L-glutamate were classified into the rank of "Minimal", Polyoxyethylene Hydrogenated Castor Oil (60 E.O.), Polyoxyethylene Sorbitan Monolaurate (20 E.O.), Polyethyleneglycol Monolaurate (10.E.O.) and Polyoxyethylene Octylphenylether (10 E.O.) into the rank of "Minimal to Mild", Sodium N-Lauroyl Sarcosinate (30% solution) and Sodium Polyoxyethylene laurylether Sulfate (2 E.O.: 27% solution) into the rank of "Mild to Moderate" and Sodium Lauryl Sulfate and Benzalkonium into the rank of "Severe". Dose-related and valid values for Sodium Hydrogenated Tallow-L-glutamate were obtained at only two of the five testing facilities in both the preliminary and main studies, therefore, the above values were calculated only from the data obtained by the two testing facilities. As a result, the coefficient of variation of Sodium Hydrogenated

Table 2. Results of the EYTEX assay

Lab	C		E		H		P		T		Mean values of the 5 Labs.		Coefficient of variation
SAMPLE	SCORE	CLASS	SCORE	CLASS	SCORE	CLASS	SCORE	CLASS	SCORE	CLASS	MEAN±SD	CLASS	(%)
S-1	12.8 RMA	Minimal	13.9 RMA	Minimal	13.5 RMA	Minimal	15.8 RMA	Min/Mld	12.9 RMA	Minimal	13.8±1.2	Minimal	8.7
S-2	16.0 RMA	Min/Mld	15.1 RMA	Min/Mld	12.3 RMA	Minimal	20.2 UMA	Mild	17.9 UMA	Min/Mld	16.3±3.0	Min/Mld	18.4
S-3	14.7 RMA	Minimal	18.5 RMA	Min/Mld	12.6 RMA	Minimal	16.8 RMA	Min/Mld	20.6 UMA	Mild	16.6±3.1	Min/Mld	18.7
S-4	22.9 RMA	Mld/Mod	11.5 UMA	Minimal	16.9 RMA	Min/Mld	15.6 RMA	Min/Mld	18.4 RMA	Min/Mld	17.1±4.2	Min/Mld	24.6
S-5	21.9 RMA	Mild	32.2 RMA	Moderate	14.0 UMA	Minimal	24.0 RMA	Mld/Mod	22.4 RMA	Mld/Mod	22.9±6.5	Mld/Mod	28.4
S-6	-	-	-	-	15.9 RMA	Min/Mld	-	-	10.1 RMA	Minimal	13.0±4.1	Minimal	31.5
S-7	-	-	41.6 RMA	Severe	32.0 UMA	Moderate	40.2 RMA	Severe	39.5 RMA	Severe	38.3±4.3	Severe	11.2
S-8	23.7 RMA	Mld/Mod	24.8 RMA	Mld/Mod	19.8 RMA	Mild	23.5 RMA	Mld/Mod	27.1 RMA	Moderate	23.8±2.6	Mld/Mod	10.9
S-9	14.7 RMA	Minimal	18.4 RMA	Min/Mld	14.4 RMA	Minimal	15.6 RMA	Min/Mld	15.7 RMA	Min/Mld	15.8±1.6	Min/Mld	10.1
S-10	29.9 RMA	Moderate	34.9 UMA	Mod/Sev	34.9 UMA	Mod/Sev	>51 UMA	Sev/Ext	29.4 UMA	Moderate	36.0±8.8	Severe	24.4

Table 3. Classification of the Draize score and the EYTEX score

Classification	Draize Score	EXTEX Score
Minimal	> 0 - 15	0 - 15
Minimal / Mild		15 - 19
Mild	> 15 - 25	19 - 22
Mild / Moderate		22 - 25
Moderate	> 25 - 50	25 - 33
Moderate / Severe		33 - 35
Severe	> 50 - 80	35 - 45
Severe / Extreme	> 80	> 45

Tallow-L-glutamate was 31.5%, which is higher than that of the other samples. With regard to Sodium Lauryl Sulfate, its blank value tended to be high at one of the five testing facilities and a valid blank value could not be obtained within the period of the validation study. In an additional study performed after the completion of the validation study, a valid blank value of Sodium Lauryl Sulfate was obtained. This value was nearly the same as those obtained at the other testing facilities in the additional study, but the value was not included in the processing of the test results, and was treated as reference data.

Figure 1 shows a graph of the dispersion of assay results across testing facilities. In this figure, the EYTEX® scores are shown on the vertical axis and the test samples from Isotonic Sodium Chloride Solution to Benzalkonium on the horizontal axis. The results shown in the figure look especially dispersed because one testing facility reported high assay values for Polyethyleneglycol Monolaurate (10 E.O.) and Sodium N-Lauroyl Sarcosinate (30% solution) and another facility reported low values for the same test samples. Excluding these two testing facilities, the results of the two test samples reported by the remaining three testing facilities nearly coincided with each other.

Regarding the other test samples, the assay results of all the testing facilities showed a

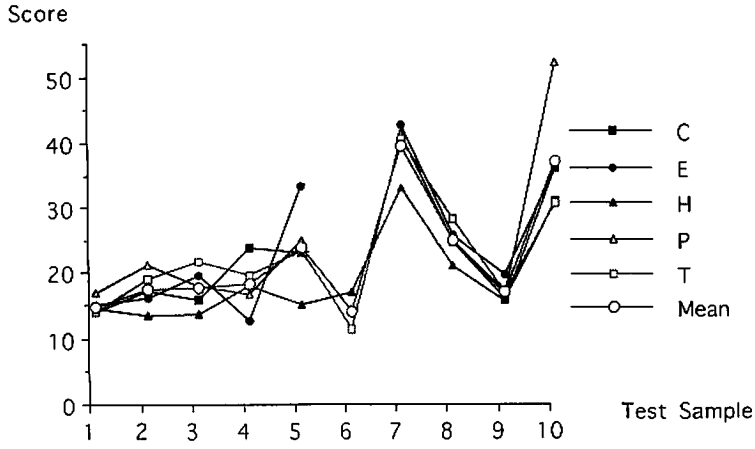

Figure 1. Results of the EYTEX assay across testing facilities.

similar tendency though there was some dispersion. In Figure 2, the correlation between the EYTEX® assay results and the *in vivo* assay results[13] is examined by plotting the EYTEX® assay results (the mean values of the five testing facilities) on the graph in which the total *in vivo* evaluation scores and the evaluation scores of the cornea, the iris and the conjunctiva reactions caused by each of the test samples have been shown in accordance with the passage of time from 0h to 72h. As the EYTEX® method is a test system designed to correlate with the *in vivo* results at the 24h point, the EYTEX® scores of each test sample were plotted against the *in vivo* results at the 24h point. Test samples showing low scores in the *in vivo* assays, from Isotonic Sodium Chloride Solution to Polyethyleneglycol Monolaurate (10 E.O.) (Figure 2), produced substantial reactions in the EYTEX® method, exhibiting values as high as 15 in this method. Test samples, giving positive reactions in the *in vivo* assays, such as Sodium Lauryl Sulfate, showed slightly higher values in the EYTEX® method than for the milder substances.

Table 4 shows the correlation coefficients between the *in vivo* assay results and the EYTEX® assay. Correlation results between the EYTEX® and Draize data for all ten test substances are shown in Figure 3 and those for eight substances (excluding Sodium Hydrogenated Tallow-L-glutamate and Polyoxyethylene Octylphenylether (10 E.O.)) are shown in Figure 4. Correlation to four kinds of scores obtained by Draize tests are depicted in these figures. Those are scores for corneal, iris and conjunctiva and maximal average Draize total score (MAS) for the following three parameters of the Draize test: the point showing the maximum value, the scores at 24h after the application of the test substances and the area under the curve (AUC) calculated from the data from day 0 to day 7. The scores obtained by the EYTEX® method were used without any transformation. There were two samples for which were not compatible with the EYTEX® method. Therefore, two correlation coefficients were calculated, one based on all ten samples and the other on the eight samples excepting those two mentioned. As a result, the correlation coefficients based on the 10 samples was 0.5. However, in the comparison based on the 8 samples the correlation coefficient was 0.7 except for the low degree of correlation in the evaluation scores of the iris reaction, which contributes only a small percentage in the evaluation scores of the Draize method. The correlation coefficient based on the evaluation scores of the conjunctival reaction at the 24h point in particular was as high as 0.955.

Table 5 shows the respective numbers of the samples which have been classified into three

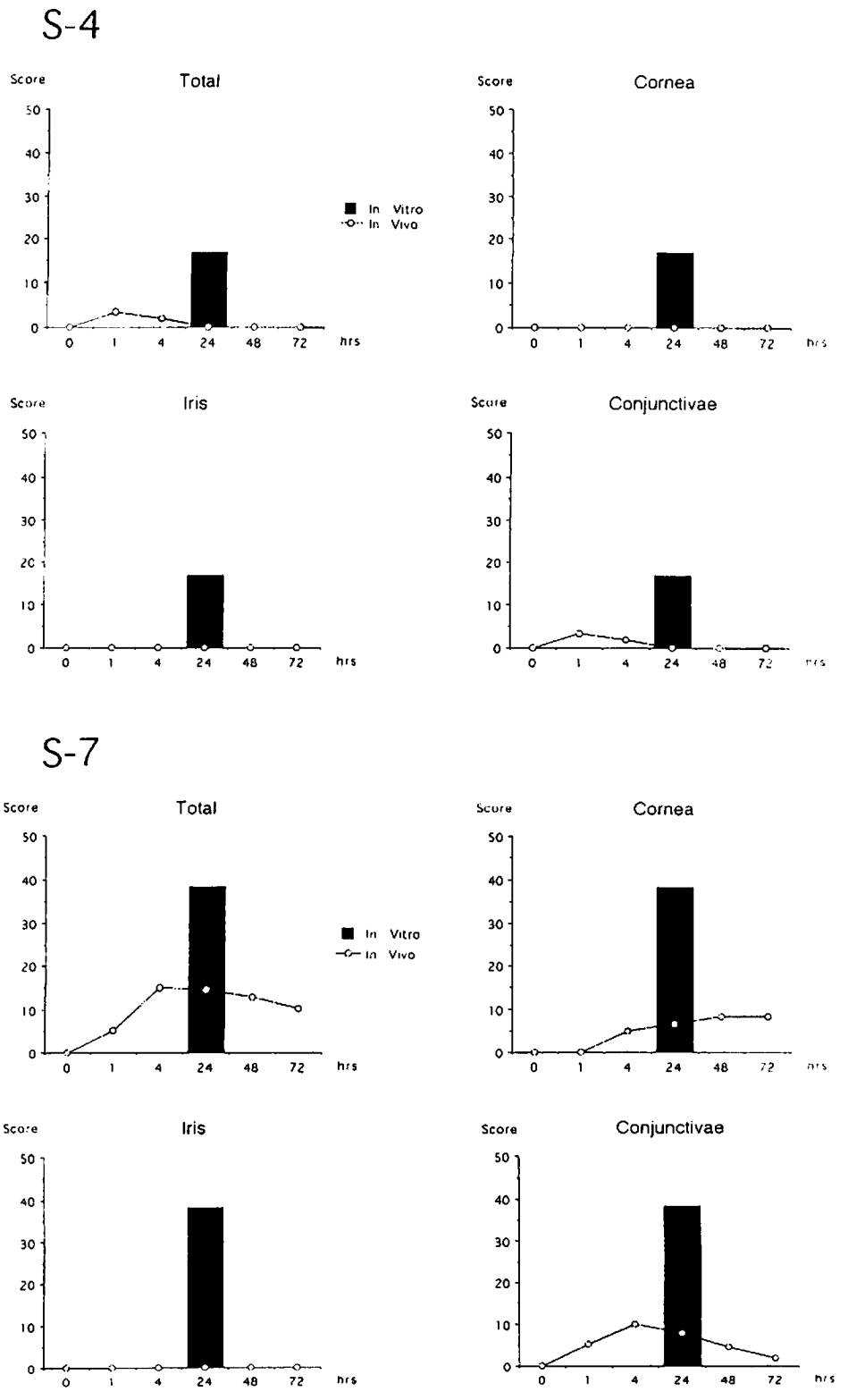

Figure 2. Correlation between the EYTEX results (at 24h point) and the Draize scores.

Table 4. Correlation coefficient between EYTEX and Draize Result

Valuation		Correlation coefficient (1)	Correlation coefficient (2)
Total score	(Max)	0.481	0.710
Cornea score	(Max)	0.486	0.661
Iris score	(Max)	0.184	0.559
Conjunctiva score	(Max)	0.564	0.884
Total score	(24h)	0.533	0.703
Cornea score	(24h)	0.523	0.636
Iris score	(24h)	-0.271	-
Conjunctiva score	(24h)	0.499	0.955
Total score	(AUC)	0.465	0.657
Cornea score	(AUC)	0.463	0.632
Iris score	(AUC)	0.219	0.559
Conjunctiva score	(AUC)	0.523	0.767

(1) S-1~S-10

(2) except S-6,S-9

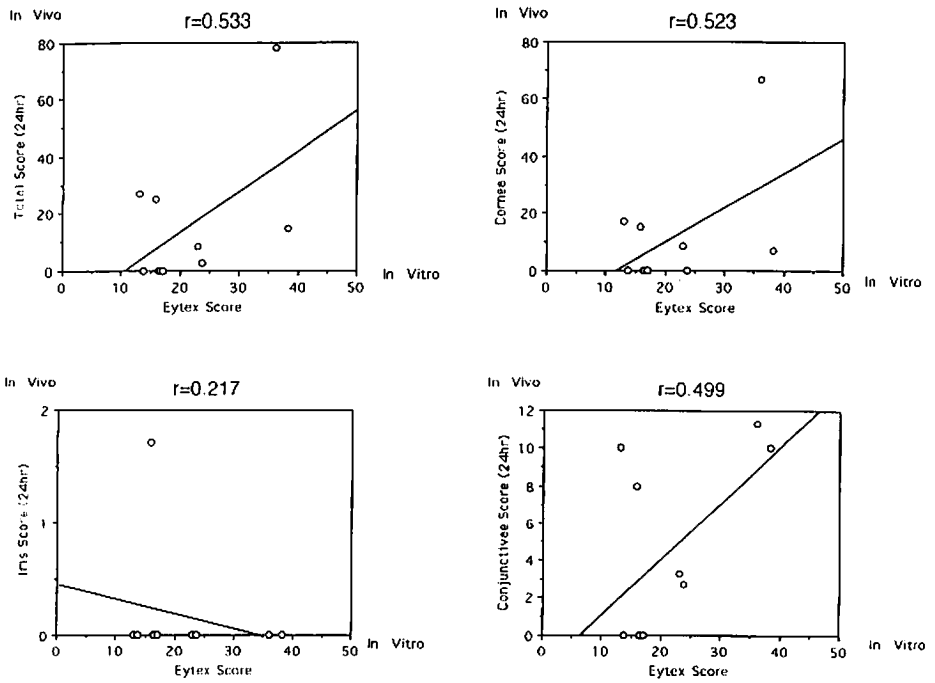

Figure 3. Correlation between the EYTEX and the Draize results for all test substances.

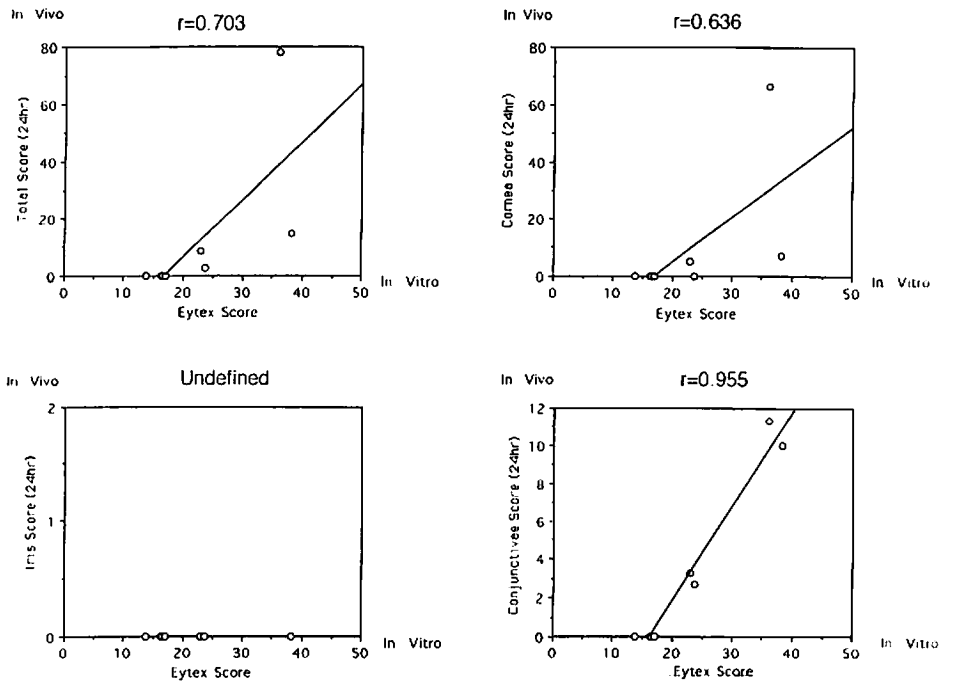

Figure 4. Correlation between the EYTEX and the Draize results for eight test substances.

Table 5. Correlation of the *in vivo* and the *in vitro* results classified into three ranks.

in vivo \ in vitro	Minimal / Mild	Mild / Moderate	Moderate / Severe
Non Irritant	4		
Slightly Irritant		2	
Moderate / Severe / Extreme	2		2

Mann - Whitney test

Correlation = (A+0.5B)/(A+B+C) x 100

A; # of samples with full correlation = 8
B; # of samples with partial correlation = 0
C; # of samples with no correlation = 2

Correlation = (8+0.5x0)/(8+0+2)x100 = 80%

ranks of irritation based on the assay results obtained by this validation study, with the *in vivo* values on the vertical axis and the *in vitro* values on the horizontal axis. The degree of correlation between the *in vivo* results and the *in vitro* results calculated from the values

given in this Table by the Mann-Whitney test was 80%.

DISCUSSION

Test substances from Isotonic Sodium Chloride Solution to Polyethyleneglycol Monolaurate (10 E.O.) (samples 1 through 4), which showed a low degree of irritation in the *in vivo* assay, showed EYTEX® scores ranging from 13.8 to 17.1. These substances were classified to be "Minimal" or "Minimal/Mild" by the EYTEX® protocol. This is because the EYTEX® method has a higher sensitivity than the *in vivo* method and because the UMA and RMA methods used for this study have a characteristic that they give a score to a sample having a score of 0 in the *in vivo* assay. An alternative protocol of EYTEX® (HSA method), which has not been used in this study, is a favorable method for assays of minimal irritants such as cosmetics since it has been developed to classify samples with a score of 15 or less in more detail.

The coefficients of variation of Polyethyleneglycol Monolaurate (10 E.O.) and Sodium N-Lauroyl Sarcosinate (30% solution) were slightly higher than those of the other samples because one testing facility gave relatively higher scores and another testing facility reported lower scores than the other facilities, as shown in Figure 1. However, the other three testing facilities gave nearly the same scores.

Regarding Sodium Hydrogenated Tallow-L-glutamate, dose-related and valid data could be obtained at only two of the five testing facilities. The problem lies in the solubility of the sample. It seems that the sample precipitated within the membrane forming solid particles which blocked the minute pores of the membrane, so that the sample did not interact with the protein matrix. As a result, the data for Sodium Hydrogenated Tallow-L-glutamate dispersed, making it impossible to form a dose-response curve. Consequently, the coefficient of variation of the sample was as high as 31.5%. Even at the two testing facilities where data for Sodium Hydrogenated Tallow-L-glutamate could be obtained, the reaction to the sample tended to cease during the course of the assay. Therefore, it is concluded that there was a problem in the handling of the sample or its solubility.

The data of Sodium Lauryl Sulfate were not judged valid at one of the testing facilities because the blank value in its assay tended to be high. The testing facility performed an additional study after the completion of the validation study, giving nearly the same data as that obtained at the other four testing facilities. This testing facility had previously performed an assay with the EYTEX® method, and at that time it obtained valid values without any problems. Therefore, as this was not a problematic sample, it is likely that the problem lies in technician error or a defect in the membrane used in the blank test.

By the *in vitro* test Polyoxyethylene Octylphenylether (10 E.O.) was classified into the rank of "minimal to mild", which is a significantly lower rank than in the *in vivo* assay. This seemed to be caused by its chemical structure having a long ethoxyl group, which is unsuitable in the EYTEX® assay because the reactions are easily inhibited by this structure. Thus, the correlation between the EYTEX® assay results and the *in vivo* assay results were also calculated excluding the data of Sodium Hydrogenated Tallow-L-glutamate and Polyoxyethylene Octylphenylether (10 E.O.).

The correlation coefficient calculated from the comparison of the *in vitro* and iris scores, which accounts for only a small percentage of the total Draize score, was the lowest, indicating low predictivity for iris responses. It should be noted that these test substances produced minimal iris responses. The correlation coefficient calculated from the MAS, the evaluation scores of the cornea and the conjunctival reactions to all ten samples was approximately 0.5 for the following three

parameters: maximum scores, scores at the 24h point and the AUC values. These low correlations were based on calculations which included the assay data of Sodium Hydrogenated Tallow-L-glutamate and Polyoxyethylene Octylphenylether (10 E.O.).

The correlation coefficient calculated from assay results for the eight samples that did not appear to interfere with the test indicated a low correlation with iris reactions, but correlation coefficients of 0.6 or more for the other *in vivo* scores. The correlation coefficient calculated from *in vitro* and conjunctival scores was particularly high, 0.884 for the maximum scores, 0.955 for the value at 24h and 0.767 for the AUC value. As the EYTEX® method is a test system designed to put an emphasis on the corneal reaction, the correlation determined by the corneal reaction was expected to be high. However, it was found that the correlation coefficient calculated from the conjunctival reaction was higher. All of the samples used in this study belong to a specific category of chemicals, a group of surfactants, and their reactions tend to be inhibited by proteins. It is therefore considered that the samples showed the acute conjunctival reaction rather than the corneal reaction.

The correlation between the EYTEX® results and the *in vivo* results was examined in relation to irritation ranks by the Mann-Whitney test. Three ranks were used to score the degree of the reactions to irritants for both the in vitro and in vivo assays, taking into consideration the tendency for the EYTEX® method to have higher sensitivity and give somewhat higher scores than the in vivo method in assaying minimal irritants. As a result, eight of the ten samples fell within the same irritation ranks so that the correlation coefficient was 80%. It was previously mentioned that Sodium Hydrogenated Tallow-L-glutamate and Polyoxyethylene Octylphenylether (10 E.O.) were not compatible with the EYTEX® assay. Consequently, regarding the eight samples for which assay results were valid, their irritation ranks corresponded with those obtained by the *in vivo* assay.

The EYTEX® method has a merit that it can assay substances with a wide variety of descriptions, including combination drugs and preparations, by a relatively simple procedure with sound intra-and inter-laboratory reproducibility. However, it became apparent from earlier works that there were certain substances for which this method can not be applicable. These were 1) surfactants or preparations containing large quantities of surfactants, 2) practically insoluble substances that are solidified after having been pipetted into the membrane and plug the membrane pores, 3) substances with many ethoxyl groups in their structure, and 4) samples containing a manganese purple color. These were the reasons why valid results could not be obtained for two of the ten samples in this validation study. However, regarding the remaining 8 samples, the variability between the five testing facilities was small and the correlation coefficients were high. The degree of correlation determined by irritation ranks was as high as 80%.

We conclude that the EYTEX® method is a promising alternative method to the Draize test. The second phase validation of this method is planned using a wider range of cosmetic ingredients.

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