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In Vitro Battery Test System for Predicting Eye Irritancy

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SUMMARY

Based on the hypothesis that irritation is due to damage of the cellular plasma membrane and cellular proteins, we designed an in vitro battery test system for predicting eye irritancy. The system consists of 3 tests, a hemoglobin denaturation (HDR) test to evaluate the protein denaturation factor, and tests of red blood cell (RBC) lysis and liposome lysis to evaluate the cellular plasma membrane destruction factor. Multi-regression analysis of the data obtained yielded equations for predicting Draize eye irritation scores. A combination of HDR and liposome tests gave the highest correlation to corneal Draize score (r=0.922), while combination of HDR and RBC tests gave the best correlation to total Draize score (r=0.941). These correlations are sufficiently good that this in vitro battery test system should represent a practical alternative to the in vivo Draize test for predicting eye irritancy of chemicals.

INTRODUCTION

The Draize eye irritation test[1] has been criticized from the viewpoint of animal welfare in recent years. Thus, a number of in vitro test systems for predicting eye irritancy have been developed such as the EYTEX test system[2], red blood cell test system[3], albumin denaturation test[4], and tests with SIRC and HeLa cells[5], CAM of fertilized egg[6] and liposomes[7]. We also developed a quantitative evaluation method using a hemoglobin denaturation (HDR) method[8].

In our previous report[9], seven *in vitro* tests used to predict eye irritancy (EYTEX, SIRC, HeLa, CAM, liposome, red blood cell and hemoglobin denaturation test system) were applied to 12 surfactants and the results were subjected to multivariate factorial analysis, and major factors for the prediction of the eye irritancy were clarified; these were destruction of cellular plasma membrane system and protein denaturation[10][11]. In the present study, on the basis of these previous results, we adopted the HDR method to measure protein denaturation and the RBC method and/or liposome method to evaluate cellular plasma membrane destruction in an attempt to improve the accuracy of prediction by multivariate analysis[8][12].

12 surfactants (Table 1) were examined with the three in vitro tests, and the results were subjected to multi-regression analysis to yield equations for predicting Draize eye irritation scores.

MATERIALS AND METHODS

1) In vivo test (Draize test)

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Table 1. Surfactants tested.

Type of surfactant	Surfactant	Abbreviation
Cationic	Hexadecyl pyridinium chloride	C1
	Octadecyl trimethyl ammonium chloride	C2
	Dodecyl trimethyl ammnomium chloride	C3
Anionic	Alkyloyl taurate	A1
	Sodium dodecyl sulfate	A2
	Acyl glutamate	A3
	POE dodecyl ether sulfate	A4
	Sodium caseinate	A5
Nonionic	POE (10) octyl phenyl ether	N1
	POE (20) octyl phenyl ether	N2
Amphoteric	Alkyl betaine	AM1
	Alkyl amido betaine	AM2

The accumulated *in vivo* Draize test data were taken from a previous report[5] for comparison with the results of the *in vitro* tests.

2) Hemoglobin denaturation test

Hemoglobin was dissolved in the standard phosphate buffer (pH 6.86) at a concentration of 0.05% (w/w). Surfactants were diluted with ion-exchanged water to make 2.0% (w/w) solutions, and from each solution, a series of 12 two-fold dilutions was prepared. Aliquots (100 μl) of each dilution were placed in 8 lines of a 96 well micro plate (Sumitomo Bakelite Co., Ltd., Tokyo, Japan). An equal amount of hemoglobin solution in the buffer was added to each well of 4 lines, and buffer solution alone was added to the other 4 lines. The microplate was incubated for 5 minutes at 25°C and optical absorbance at 418 nm was measured with an EIA reader. The data (n=4) were processed in accordance with the following equation (Equation 1) and the hemoglobin denaturation ratio (HDR) at each concentration was calculated.

$$HDR=100-\{Abs(SHB)-Abs(SB)\}/\{Abs(WHB)-Abs(WB)\}*100\ (\%) \quad \text{(Equation 1)}$$

where Abs(SHB): Absorbance of surfactants mixed with hemoglobin/buffer solution,
Bs(SB): Absorbance of surfactants mixed with buffer solution,
Abs(WHB): Absorbance of ion-exchanged water mixed with hemoglobin/buffer solution.
Abs(WB): Absorbance of ion-exchanged water mixed with buffer solution

Hemoglobin denaturation test data at 2%, 1%, 0.125%, 0.063% (concentrations essential to predict eye irritation by regression analysis) were taken for the use[8].

3) Liposome test

Bovine eyes were obtained from a slaughterhouse and the cornea of each eye was carefully removed with a scalpel knife and fine scissors. Lipid extracts were obtained according to the method of Bligh and Dyer[13]. To 50 sheets of isolated cornea, minced with scissors and homogenized in 200 ml of saline using an electric homogenizer, 750 ml of chloroform-methanol 1:2, v/v) was added, and mixed in a homogenizer. The homogenate was filtered, and chloroform and saline were added. The solution was centrifuged at 1430 ×g for 10 minutes, after which the lower layer (chloroform extract) was recovered and evaporated to dryness. The lipid residue was then dissolved in chloroform in a small conical flask. A sample of the extract was digested with perchloric acid and sulfuric acid, and the organic phosphorus derived from the phospholipid was converted to phosphoric acid, which was further treated with ammonium molybdate. The content of phosphorus was

determined spectrophotometrically at 750 nm, on the basis of the formation of phosphomolybdate. A sample of the chloroform extract containing 13 mM phosphorus was then completely evaporated under a nitrogen gas flow to produce a thin film. The dried lipid film inside the flask was then dispersed in 0.3 mM glucose solution (900 ml) plus 100 mM-aqueous 4-methylumbelliferyl phosphate (Um-P) solution (100 ml) and the mixture was heated at 50°C for 5 minutes in a water-bath, followed by agitation using a Vortex mixer. The heating and mixing procedure was repeated twice, and the emulsion was stored in a refrigerator for 1 hour. The emulsion was then suspended in ice-cold 0.02 M Tris-HCl buffer solution (pH 7.4) and subjected to ultracentrifugation at 123,400 ×g at 4°C for 10 minutes. The pellet was washed by ultracentrifugation with Tris-HCl buffer solution three times to remove unbound marker and finally suspended in 10 ml of cold buffer solution. The formation of liposomes was confirmed by electron microscopy after negative staining with 2% sodium phosphotungstate (pH 6.5)[14]. A mixture containing the test sample solution at an appropriate concentration (100 ml), the liposomal suspension (100 ml) and 0.02M Tris-HCl buffer solution (pH 7.4, 800 ml) was incubated at 37°C for 2 hours, then 300 ml was transferred to a test tube containing 2.7 ml alkaline phosphatase solution (0.4 U/ml) and incubated at 25°C to convert the released marker (Um-P) into an intense fluorophore (4-methylumbelliferone). The fluorescence intensity was then measured with excitation at 340 nm and emission at 448 nm, and the degree of degradation of the liposomes was calculated. The average value of Um-P50 (the concentration of test substance at which 50% of Um-P was released from the liposomes) in triplicate assays was determined for each test material.

4) Red blood cell test

Red blood cells from guinea pigs were isolated by centrifugation (1430 ×g, 10 min), and washed several times with phosphate-buffered saline (PBS; pH 7.4). The thoroughly washed cells were then stored in PBS at 4°C. Before use, the cells were adjusted to 2.5% (v/v) with PBS. For the assay, 2 ml of the red blood cell suspension was mixed with 2 ml of sample solution in a test tube and incubated for 60 min at 37°C. The incubated samples were centrifuged (360 ×g, 5 min) to remove intact cells. The resulting supernatant (1 ml) was diluted 5 times with PBS and the absorbance at 540 nm was measured with reference to a blank. The total release of hemoglobin from red blood cells was set at 100%, and the dose-dependent release was plotted to obtain the dose causing 50% cell lysis.

5) Statistical analysis

Multi-regression analysis (stepwise method) was applied to analyze the results of the three *in vitro* tests. Two combinations were examined, the HDR method and liposome method, and the HDR method and red blood cell method. The calculations were performed using the Lotus 1-2-3 program with an add-in package program for statistical analysis (Lotus 1-2-3 Multivariate analysis v 1.0) provided by Audemain, Tokyo, Japan.

RESULTS

The data obtained in the three *in vitro* test systems are summarized in Table 2. The results of the multi-regression analysis are summarized in Table 3–6. The equations obtained give predictions that correlate highly with the Draize test values (Figure 1–4).

For comparison, the correlation coefficients of individual *in vitro* test systems and the Draize scores are shown in Table 7, together with those of the new battery systems.

(1) Combination of HDR test and liposome test

The corneal score in the Draize test (Dc) could be estimated well (r=0.922, Figure 1,

Table 2. Results of assays.

Test system	Draize score		HDR (%)				LIPO (μg/ml)	RBC (μg/ml)
Surfactant	Corneal	Total	2%	1%	0.125%	0.063%		
C1	45.0	70.0	45.212	48.205	50.548	50.449	18	31
C2	36.7	60.3	38.372	36.754	37.318	36.299	26	28
C3	32.5	53.0	37.494	22.144	0.0	0.0	720	1000
A1	21.7	42.0	28.593	20.709	14.721	7.535	72	54
A2	18.3	35.0	31.222	37.406	15.033	5.279	120	70
A3	8.6	24.9	36.460	41.755	0.0	0.0	1000	1000
A4	6.7	22.0	18.223	22.312	2.473	0.0	190	260
A5	0.0	2.0	0.0	0.0	0.0	0.0	1000	1000
N1	20.0	34.7	0.0	7.025	0.0	0.0	140	160
N2	0.0	8.7	2.167	9.980	0.0	0.0	1000	250
AM1	21.7	40.2	24.334	28.557	0.0	0.0	560	760
AM2	10.8	27.5	17.803	20.762	0.0	0.0	1000	370

*Abbreviations; HDR, hemoglobin denaturation test system; LIPO, liposome test system; RBC, red blood cell test system.

Table 3. Results of multiple linear regression analysis (combination of LIPO and HDR). Criterion variable, corneal score of the Draize eye-irritation test; Explanatory variables, LIPO and HDR.

Variable	Regression Coefficient	F-Value
LIPO	−0.011	3.391
HDR at 2.000%	0.896	8.217*
HDR at 1.000%	−0.659	3.595
HDR at 0.063%	0.341	3.620
Constant	16.769	

Multiple regression coefficient: 0.922

Analysis of Variance	Sum of Square	Degree of Freedom	Mean Square	F-Value
Total	2294.590	11		
Regression	1950.820	4	487.705	9.931**
Residual	343.770	7	49.110	

Suggest: "*, P<0.05; **, P<0.01."
F-test of each variable indicates the contribution of the variable to the regression.

Table 3) using the following equation:

$$Dc=16.769-0.011*LIPO+0.896*HDR\ at\ 2\%-0.659*HDR\ at\ 1\%+0.341*HDR\ at\ 0.063\%$$ (Equation 2)

Table 3 shows that HDR at 2% had the highest F-value (P<0.05), indicating that the HDR at 2% makes the major contribution to the prediction of corneal irritancy. Although the F-values of the other variables were not significant, the combined F-value was significant (P-<0.01), suggesting that the variables chosen to establish the equation did contribute to the prediction of corneal Draize score.

On the other hand, the total score in the Draize test (Dt) could be estimated using the following regression equation (r=0.880, Figure 2, Table 4):

$$Dt=25.615-0.019*LIPO+0.802*HDR\ at\ 2\%$$ (Equation 3)

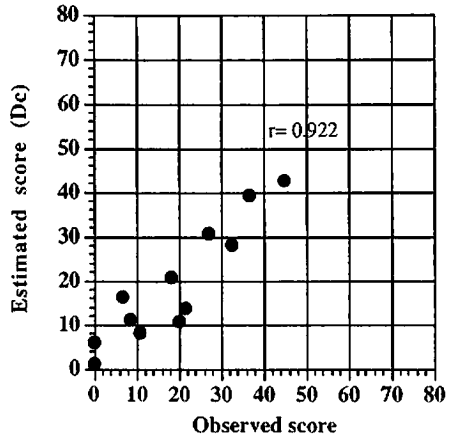

Fig. 1. Correlation between observed corneal Draize score and that estimated by Equation 2.

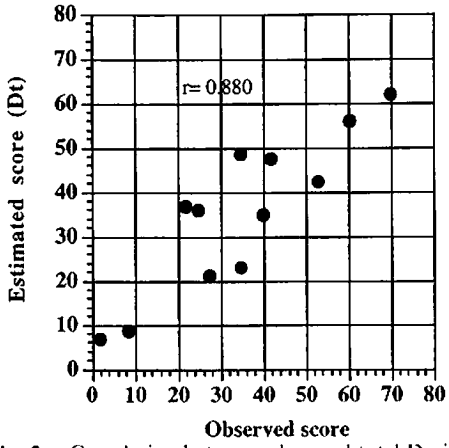

Fig. 2. Correlation between observed total Draize score and that estimated by Equation 3.

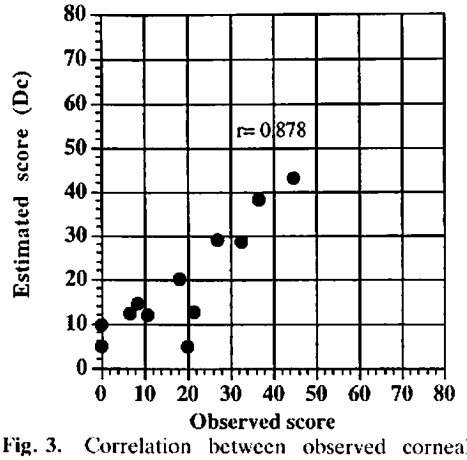

Fig. 3. Correlation between observed corneal Draize score and that estimated by Equation 4.

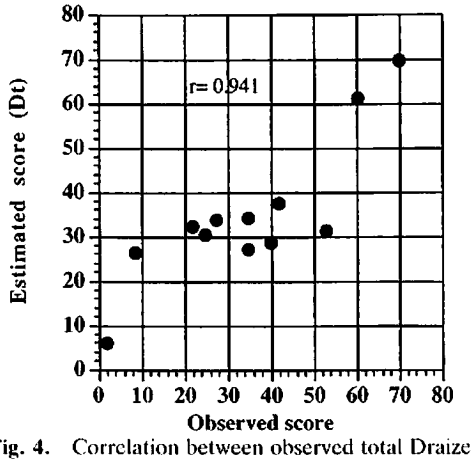

Fig. 4. Correlation between observed total Draize score and that estimated by Equation 5.

Table 4. Results of multiple linear regression analysis (combination of LIPO and HDR). Criterion variable, is total score of the Draize eye irritation test; Explanatory variables, LIPO and HDR.

Variable	Regression Coefficient	F-Value
LIPO	−0.019	2.423
HDR at 2.000%	0.802	3.731
Constant	25.615	

Multiple regression coefficient: 0.880

Analysis of Variance	Sum of Square	Degree of Freedom	Mean Square	F-Value
Total	4373.163	11		
Regression	2341.993	2	1693.059	15.438**
Residual	987.044	9	109.672	

Suggest: "*, $P<0.05$; **, $P<0.01$."
F-test of each variable indicates the contribution of the variable to the regression.

Although none of the variable individually made a significant contribution in terms of the F-value, the combined F-value was significant ($P<0.01$).

(2) Combination of HDR test and RBC test

The corneal score in the Draize test could be estimated by the following regression equation ($r=0.878$, Figure 3, Table 5).

$$Dc=9.504+0.898*HDR \text{ at } 2\%-0.667*HDR \text{ at } 1\%+0.494*HDR \text{ at } 0.125\% \quad \text{(Equation 4)}$$

The HDR at 2% and HDR at 0.125% contributed significantly ($P<0.05$) to the regression, and the combined F-value was also significant ($P<0.01$), indicating that the variables of the equation contributed to the prediction of corneal Draize score. In this

Table 5. Results of multiple linear regression analysis (combination of RBC and HDR). Criterion variable is corneal score of the Draize eye-irritation test; Explanatory variables, RBC and HDR.

Variable	Regression Coefficient	F-Value
HDR at 2.000%	0.898	6.154*
HDR at 1.000%	−0.667	2.748
HDR at 0.125%	0.494	6.827*
Constant	9.504	

Multiple regression coefficient: 0.878

Analysis of Variance	Sum of Square	Degree of Freedom	Mean Square	F-Value
Total	2294.590	11		
Regression	1770.175	3	590.058	9.001**
Residual	524.415	8	65.552	

Suggest: "*, $P<0.05$; **, $P<0.01$."
F-test of each variable indicates the contribution of the variable to the regression. RBC parameter did not appeared because its F-value was too small to be significant.

Table 6. Results of multiple linear regression analysis (combination of RBC and HDR). Criterion variable is total score of the Draize eye-irritation test; Explanatory variables, RBC and HDR.

Variable	Regression Coefficient	F-Value
RBC	−0.025	8.708*
HDR at 2.000%	0.671	10.370**
HDR at 0.125%	−1.774	3.362
HDR at 0.063%	1.960	4.742
Constant	30.877	

Multiple regression coefficient: 0.941

Analysis of Variance	Sum of Square	Degree of Freedom	Mean Square	F-Value
Total	4373.163	11		
Regression	3871.409	4	967.852	13.503**
Residual	501.753	7	71.679	

Suggest: "*, $P<0.05$; **, $P<0.01$."
F-test of each variable indicates the contribution of the variable to the regression.

case, RBC parameter was not used as a variable for the prediction.

Nevertheless, total Drize score was predicted well by the combination (r=0.941, Figure 4, Table 6):

*Dt=30.877−0.025*RBC+0.671*HDR at 2%−1.774*HDR at 0.125%+1.960*HDR at 0.063%*

(Equation 5)

The RBC and HDR at 2% significantly contributed to the regression, and the combined F-value was also significant at ($P<0.01$).

DISCUSSION

In the combination of HDR and LIPO tests, HDR at 2% played the most important role in the estimation of corneal Draize score since its F-value was the higher. This indicated that corneal irritation arises primarily from protein denaturation at a high concentration of test agent, and that membrane destruction plays little role. In the case of estimation of the total Draize score, the combined F-value was highly significant, although the F-values of individual parameters were so low that we cannot rule out the possibility that their regression coefficients were zero. It is possible that total score is determined by complicated events, and that two factors are insufficient for an adequate explanation.

With the combination of HDR and RBC tests, surprisingly, only HDRs contributed to the estimation of corneal Draize scores and RBC did not appear in the equation. This result supports the above suggestion that corneal irritation is predominantly due to protein denaturation. If this is the case, however, individual correlations of HDRs to Drtaize corneal score should be high. In fact, HDRs showed no such high correlation to corneal Draize score, although multi-regression using HDRs gave a better result (Table 7). This means that HDR measurement at a single concentration is not sufficient to represent protein denaturation which may be a complex process. We have previously suggested that hemoglobin may be denatured via two mechanisms by surfactants[8]. A better understanding of protein denaturation processes is needed before any definite conclusion can be reached.

HDR at 2% and RBC both played important roles for the estimation of total Draize score, and F-values were large enough to confirm their significance. This suggests that total Draize score is influenced by both protein denaturation and cellular plasma membrane destruction, notwithstanding the comments in the case of HDR/LIPO combination.

Correlation coefficients of results in several previously reported *in vitro* test systems and the Draize scores are shown in Table 7 for comparison with the newly established battery system. Among the individual test systems, the CAM test system shows the highest correlation coefficients with both corneal and total Draize scores. However, the battery system of HDR and liposome tests gave a superior correlation to the corneal Draize score, and that of HDR and RBC tests was better for the prediction of total Draize score. The SIRC and HeLa cytotoxicity tests and liposome test did not show good correlations, although high correlations with corneal and total scores have been reported with data plotted on a log scale[5)7)]. These correlations seem very high compared with HDR test and even with the CAM test, but the rationality of scaling by logs is uncertain.

The new battery test systems seem to be markedly better than the previously reported tests as predictors of the Draize scores. This may be because the Draize test is more correctly reproduced by the addition of the cellular plasma membrane destruction factor.

The validity of the application of multi-regression analysis for the present purpose is dependent on the assumption that the response of eye irritation is linearly related to

Table 7. **Correlations between test results and Draize scores.**

Test system	Test condition	Correlation coefficient	
		Corneal	Total
HDR	2.000%	0.739	0.792
	1.000%	0.592	0.677
	0.125%	0.761	0.744
	0.063%	0.753	0.740
HDR (multi-regression)	2.000% 1.000% 0.125%	0.878	—
	2.000% 0.063%	—	0.861
EYTEX		0.357	0.362
SIRC		−0.693	−0.671
		(−0.816)	(−0.863)
HeLa		−0.635	−0.675
		(−0.961)	(−0.978)
CAM		0.888	0.906
LIPO		−0.677	−0.652
RBC	human	−0.458	−0.468
	rabbit	−0.631	−0.635
	guinea pig	−0.684	−0.649
	rat	0.353	−0.358
HDR+LIPO		0.922	0.880
HDR+RBC rat		0.878	0.941

protein denaturation and cellular plasma membrane destruction. It is not clear whether this is the case, but the high correlation coefficients obtained do appear to justify the combination of two or more test systems in a battery system to predict eye irritation. The results indicate that the hemoglobin denaturation test is predominant as a predictor of corneal score, whereas the HDR and RBC tests have equal weight for prediction of the total Draize score, suggesting that both protein denaturation and cellular plasma membrane destruction influence the total Draize score. This seems a reasonable conclusion. The correlation coefficients obtained in the present study were higher than those reported previously from multi-regression analysis of results of the HDR test, and we consider that they are high enough to justify the use of this *in vitro* battery system as a practical alternative to the *in vivo* Draize eye irritation test.

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Announcements

Comming Events

Title:	**The 8th Annual Meeting of the Japanese Society for Alternatives to Animal Experimentation**
Date:	November 28-29, 1994
Location:	Komaba Eminence, 2–19–15, Oohashi, Meguro-ku, Tokyo 153 Tel 03–3485–1411
Organizer:	Dr. Yukiaki Kurodo, Azabu University
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