VALIDATION AND UTILIZATION OF THE SKINTEX™ SYSTEM

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ABSTRACT

The SKINTEX Method is based on a two-compartment physico-chemical model which includes a Biomembrane Barrier in compartment one and an organized macromolecular matrix in compartment two. Test samples absorb onto or permeate through the keratin/collagen Biomembrane Barrier and then can interact with the organized macromolecular matrix. Changes in the integrity of the barrier releases a dye indicator: Changes in the matrix can alter its transparency. The sum of these two responses is read spectrophotometrically at 470nm.

An early investigation of 950 chemicals and formulations in the SKINTEX System produced results which were 89% concordance to *in vivo* Draize dermal irritation results obtained with 24-hour occluded application of test samples without abrasion and standard scoring. Alkaline materials were analyzed in a specialized SKINTEX AMA Protocol.

In this early study, the model did not distinguish nonirritant test materials and formulation with PDII(Primary Dermal Irritation Index)in the range from 0 to 1.2. A High Sensitivity Assay Protocol(HSA)was developed to amplify the changes in both compartments of this model and provide more accurate calibration of these changes. A study of 60 low irritation test samples including cosmetics, household products, chemicals and petro-chemicals distinguished nonirritants with PDII$\leq$0.7 for 26 of 30 nonirritants.

A second protocol was developed to evaluate the SKINTEX model predictability with respect to human irritation. The Human Response Assay (HRA)has been optimized based on differences in penetration and irritation responses in humans and rabbits. An additional 32 test materials with different mechanisms and degrees of dermal toxicity were evaluated by th HRA. These *in vitro* results were 86% concordant to human patch test results.

In order to further evaluate this model, a Standard Chemical Labelling (SCL) Protocol was developed to optimize this system to predict Draize dermal irritation results after a 4-hour application of the test material. In a study of 52 chemicals including acids, bases, solvents, salts, surfactants and preservatives, the SCL results demonstrated 85% concordance to Draize results for a 4-hour application of test samples on non-abraded rabbit skin.

The SKINTEX System, including three specialized protocols, provided results which demonstrated good correlation to the endpoint of dermal irritation in man and rabbits at different application times.

INTRODUCTION

Many commercial products are recognized as potential sources of dermal irritation. A multitude of chemicals in the workplace and environment have been recognized as dermal irritants. Federal agencies and commercial manufacturers must consider dermal irritation risks when developing, registering or certifying materials.

The test most widely used for predicting potential skin irritants in humans with an animal model was published by Draize et al (1944)(1). Many modifications to improve inconsistent results and interpretation have been utilized. The DOT(Department of Transportation)recommends an exposure period of four hours(CFR 1988)(2). Four hour and shorter periods are recommended by the NAS(National Academy of Science, 1977)(3).

The Draize method has been a reference procedure despite its domonstrated intra-laboratory variability in scoring and rating(Well and Scala, 1971)(4). The method is unable to identify mild or moderate irritants (5)(Philipsetal, 1972) and produces many false positives(Nixonetal, 1975). The Draize method produces results which are not comparable to results obtained in humans. It also produces irritation rankings for a series of materials which are different than irritation rankings produced by human patch testing. Similar conclusions were reached in the OECD Guidelines. The Organization for Economic Cooperation and Development(OECD)released a series of guidelines for testing of chemicals in 1981(7). The OECD dermal irritation guideline recommended 4-hour application without skin abrasion. The guideline states that extrapolation of animal results to humans has only limited value.

The SKINTEX model incorporates two compartments. The first compartment is a Biomembrane Barrier of Keratin and Collagen(8,9,10). Materials can absorb onto or permeate through this Biomembrane Barrier to a macromolecular matrix. Changes in the integrity of the first compartment due to absorption of a test sample to the keratin can release dye incorporated into this Biomembrane. In the second compartment, organized protein filaments are spatially arranged with collagen into a transparent matrix. Test samples which are chemical irritants alter the conformation and/or hydration of the protein filaments to product turbidity(see Figure 1). Lipid components are associated with small proteins and are include in the ordered matrix(see Table 1).

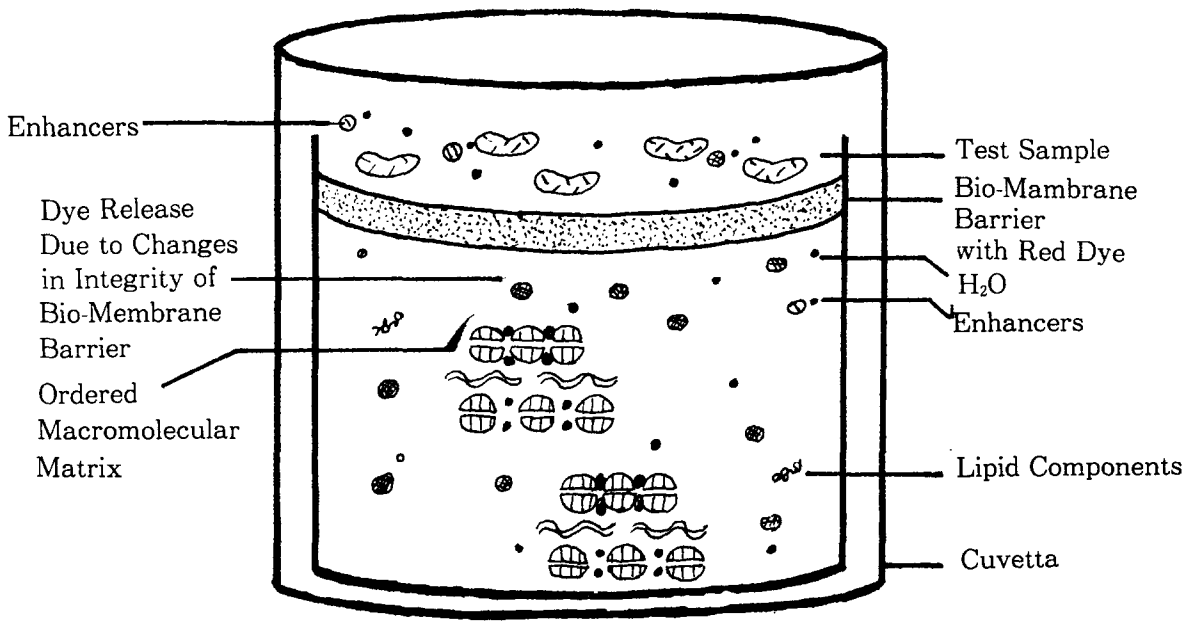

Figure I. The SKINTEX Model

The definitions relevant to this model are summarized in Table 1.

SKINTEX Definitions
Barrier Membrane Keratin and collagen cross-linked to cellulose support. Changes in integrity of Biomembrane Barrier measured by release incorporated into barrier.
Highly Ordered Macromolecular Matrix Consisting of oligomeric protein arranged spatially with collagen where interactions between large molecules stabilize the matrix and provide transparency.
Lipid Components Such as phospholipid and cholesterol are present bound to small proteins.

Table 1. SKINTEX Defintions

There is no single all-inclusive predictive model for primary dermal irritation. Therefore in developing the SKINTEX model, refinement has included optimization of rest protocols to predict an irritant response observed in human patch testing, to predict the irritant response observed after 24-hour application in rabbits (UMA), to predict the irritant response observed after 4-hour application in rabbits (SCL)and to predict the response of very low irritation test samples (HSA) accurately. These four protocols, the Upright Membrane Assay(UMA), the High Sensitivity Assay (HSA), The Standard Chemical Labelling (SCL)and the Human Response Assay (HRA)constitute the SKINTEX System (see Figure 2).

The SKINTEX System

Scientific Basis
- Alteration of a Biomembrane Barrier or Permeation through a Barrier Membrane and Interaction with a Highly Ordered Macromolecular Matrix.

Reagents and Instrumentation
- Standardized Reagent, Controls, Calibrators and Instrumentation

Protocols
- UMA:Broad Screening Protocol
- HSA:Low Irritation Samples Protocol
- AMA:Alkaline Materials Protocol
- HRA:Predict Human In Vivo Response Protocol
- SCL:Predict 4-Hour Application in Draize Dermal Test Protocol

DAQC-EX Software
- Data Analysis, Assay Performance Control for SKINTEX

Figure 2. The SKINTEX System

MATERIALS AND METHODS

The SKINTEX Method includes a Biomembrane Barrier and Biomacromolecular Matrix.

Biomembrane Barrier: A buffered salt solution at pH8.0 of 10% keratin and 1% collagen was bound to cellulose acetate with 0.1% glutaraldehyde at 25℃ for one hour. After washing the support in distilled water, Basic Red 2 was at-

tached to the keratin/collagen matrix with 0.1% glutaraldehyde for 10 minutes at 25℃ Biomembrane Barriers were stored at 4℃ and have a 180day shelf life. Circles are cut and formed into a well within a plastic disc.

Biomacromolecular Matrix: A lyophilized powder containing globulins, collagen, glycosaminoglycans, free fatty acids, amino acids, phospholipids and buffer salts is re-hydrated. The re-hydrated reagent is stable for 15 days at 4℃.

Protocols: A prototype method, the Upright Membrane Assay (UMA), was used to establish the relationship between the response or known irritants and their *in vivo* dermal irritation. This procedure permitted testing of liquids, solids and insolubles undiluted at three different doses. Samples were applied directly to the barrier matrix and inserted into the reagent. The optical density at 470nm was used to quantitate the response. A calibration system based on the irritancy (PDII) of known controls was used to establish a scoring system. The net O.D. at 470nm is read as a SKINTEX/PDII Equivalent. *In vitro* dermal irritation classes of minimal, mild, moderate and severe correspond directly to *in vivo* classes based on Draize rabbit skin scores with a 24-hour application (see Figure 3).

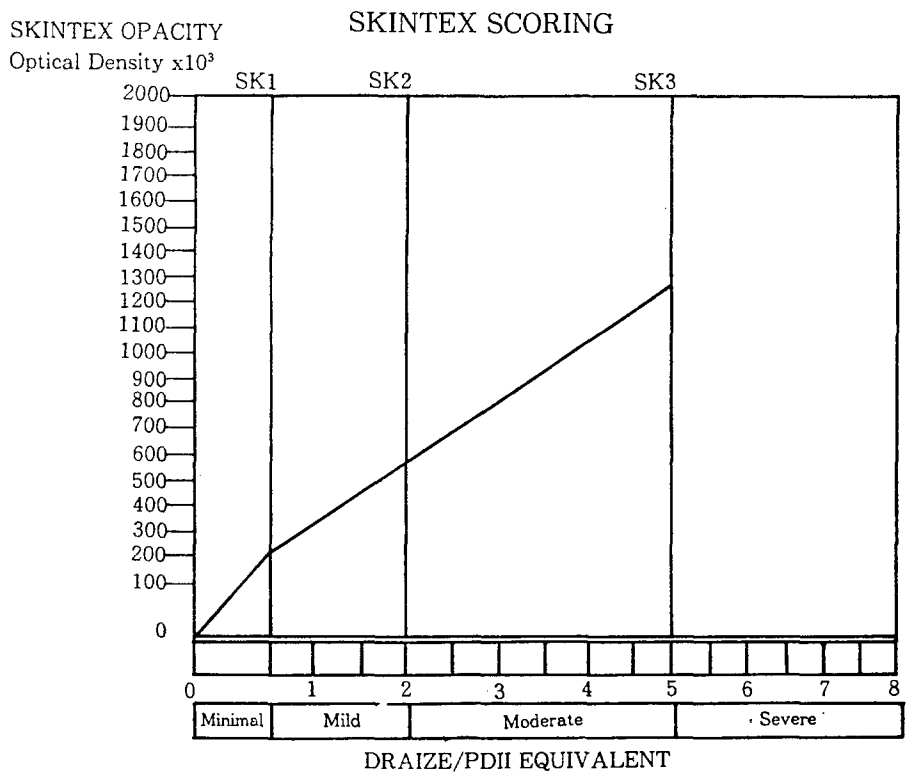

Figure 3. Scoring

The basic outline of protocols is summarized in Figure 4.

The SKINTEX
Dermal Irritation Assay

* Samples:
  Liquids, solids and insolubles can be studied undiluted
* Application:
  Test material placed undiluted onto Biomembrane Barrier held by circular disc at three doses
* Procedure:
  Biomembrane Barrier inserted into Biomacromolecular Matrix with incubation at 25℃
* Measurement:
  Optical density readings in spectrophotometer at 470nm
* Calibration and Scorings:
  Calibration with known irritants

Figure 4. Procedural Summary

The Alkaline Membrane Assay (AMA): Test samples with pH 0-2 and 12-14 cannot be analyzed in SKINTEX. A specialized protocol for alkaline materials pH9-12 has been developed which utilizes alkaline calibrators and controls for standardization and scoring(see Figure 5).

	pH Optimization	
>	pH 0-2	NQ(Dilute Into Range)
>	pH 2-9	Activated
>	pH 9-12	Nonactivated Alkaline Protocol(AMA)
>	pH 12-14	NQ(Dilute Into Range)

Figure 5. pH Optimization for SKINTEX UMA/AMA

The High Sensitivity Assay(HSA): Developed for test samples with low irritation. This assay increases the ratio of test sample to active reagent for increased sensitivity. Three calibrators with PDII of 0.2, 0.5 and 1.0 increase the accuracy in this range.

The Human Response Assay(HRA): Provides scoring and classification based on the Frosch-Kligman(18) *in vivo* scoring system. The optical density results can be analyzed to produce a human score for prescreening materials prior to human testing.

The Standard Chemical Labelling Protocol (SCL): Provides scoring and classification based on the Draize rabbit dermal test results with 4-hour application without abrasion.

SCHEME FOR EVALUATION

These protocols are incorporated into a current scheme for evaluation of test samples for Dermal Irritation. The scheme is summarized in Figure 6.

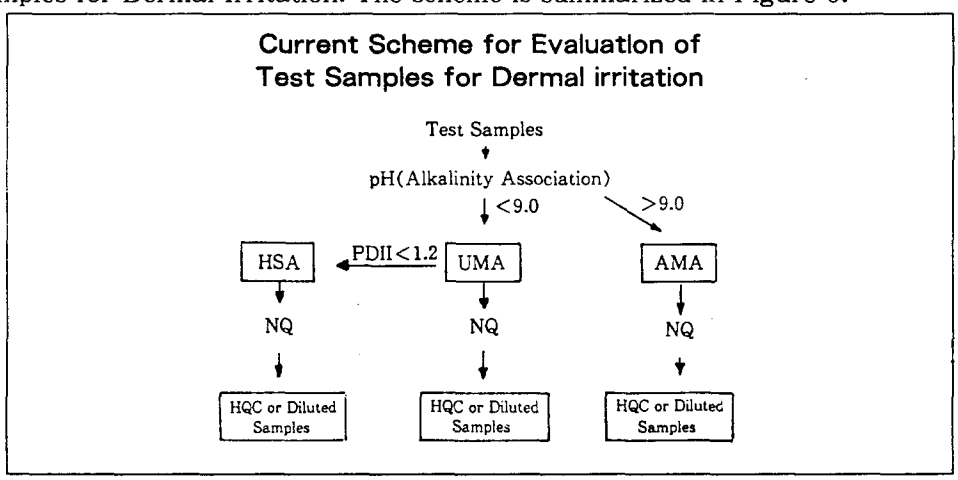

Figure 6. Current Scheme for Evaluation of Test Samples for Dermal Irritation

DATA ANALYSIS AND QUALITY CONTROL OF THE SKINTEX SYSTEM

Integral to the full utilization of these protocols is the DAQC software for data analysis and quality control. There are several key elements which must produce results in qualified ranges for assay performance. If those are obtained then test sample results can be evaluated(see Figure 7).

KEY ELEMENTS OF CONTROL FOR THE SKINTEX METHOD

1. Assay Performance
   - >Calibrators
   - >Controls
2. Qualification Sample Results
   - >Qualification Checks
     - – Blank Membrane
     - – Net Response
     - – Inhibition Check
     - – Dose Response Check
   - >Dose Response Curves
     - – Flat
     - – Increasing
     - – Nonqualification Decreasing Curves

Figure 7. Key Elements to Control the SKINTEX Method

One criteria is the calibration of each assay within standard limits. When two quality control samples are analyzed with this curve, they must produce acceptable values. This control of assay performance ensures standardized and reproducible results (see Figure 8).

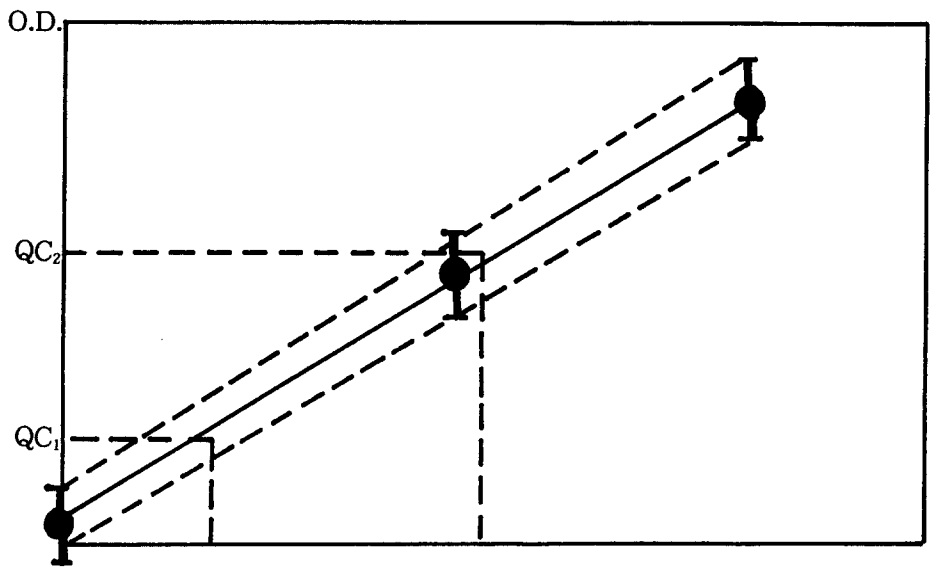

Figure 8. Assay Performance Evaluation

Finally, all test sample dose response curves must be within the linear or saturation regions of the dose response curve. Concentrations producing interference are carefully eliminated by analysis of the dose response curve. As in all *in vitro* methods, there are conditions for optimal behavior of a test sample in this system.

The SKINTEX database includes over 5300 materials from diverse industries. These materials range from nonirritants to severe irritants. Different chemical classes are represented as well as samples within a pH of 2 to 12. Of the 5300 materials tested, 90% were compatible when studied neat or at the concentration studied in the *in vivo* assay.

	SKINTEX Database
>	5300 Chemicals and Formulations
>	Range Nonirritant to Severe Irritant
>	Compatibility 90%
>	pH Range 2-12
>	Different Mechanisms Toxicity

Figure 9. Frame Scheme of Validation

The evaluation of SKINTEX has been extensive. The test method is used in over 100 laboratories worldwide. Major collaborations are in progress with S.C. Johnson, Avon and University of California, San Francisco(UCSF)(12, 13). Yves Rocher presented an evaluation of nearly 100 products in SKINTEX in June 1990 demonstrating a 90% predictive value. A study with the Food and Drug Safety Center in Japan exhibited a 100% predictive value.

RESULTS

The *in vitro* method was evaluated with respect to inter-and intra-assay reproducibility(see Figure 11).

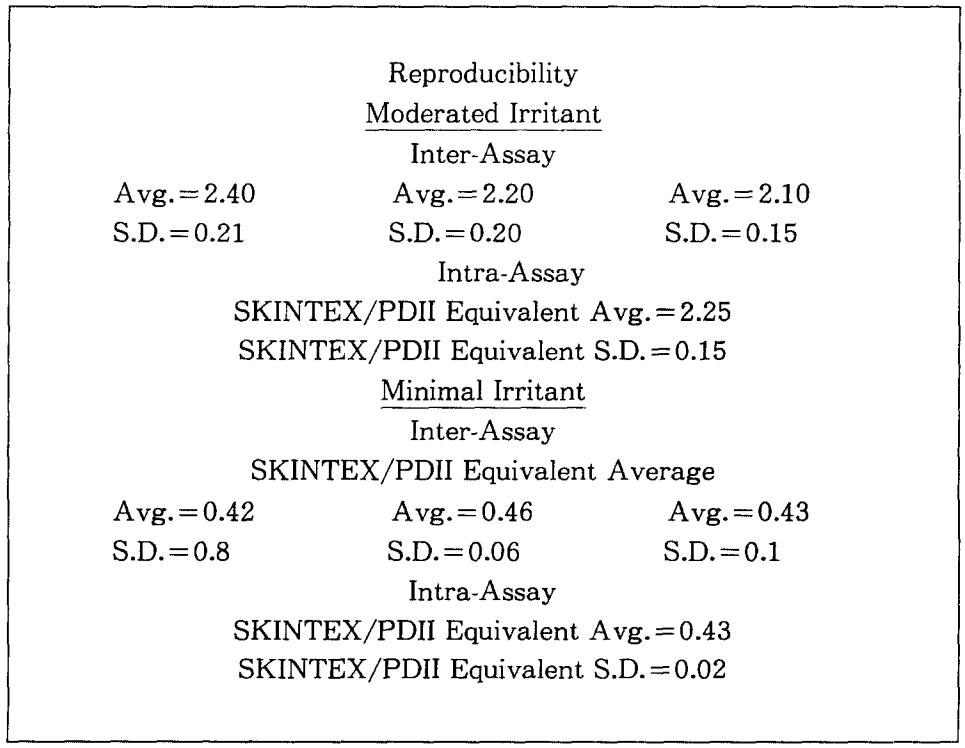

Figure 11. Reproducibility of the SKINTEX Results

Interference could be observed in the SKINTEX System. This produces a nonqualified dose response curve for the test substance. This dose response curve is qualified only if increasing doses produce flat or increasing curves(see Figures 12 and 13). Interference can be reduced by diluting the test sample. Table 2 presents interferences which have been observed in the SKINTEX Metod. For each interference, modifications of the protocol can permit re-analysis of the test material and qualification of the dose response curve.

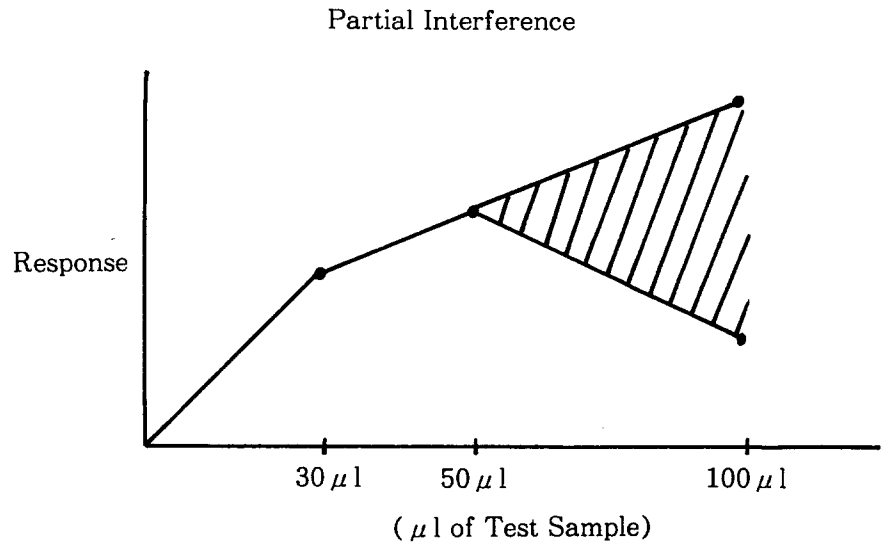

Figure 12. Partial Interference in the SKINTEX Model

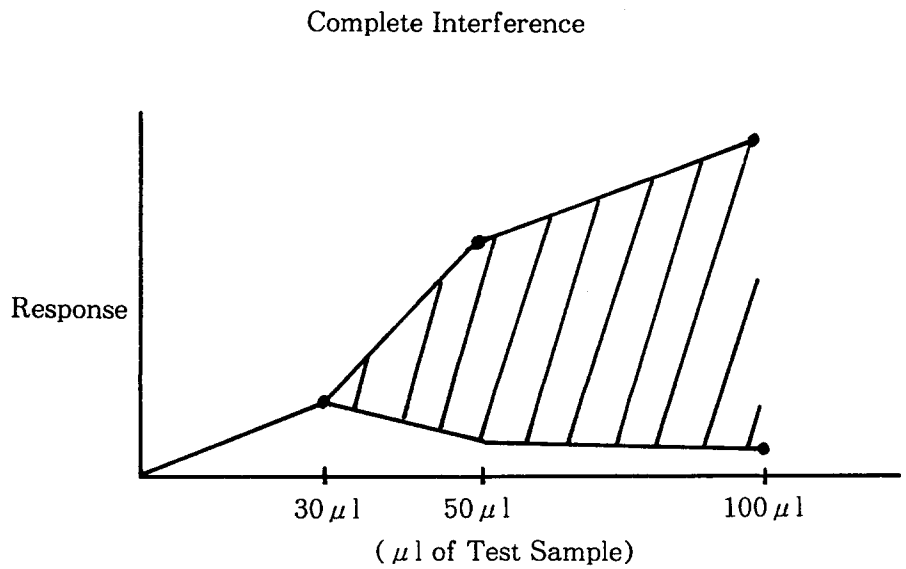

Figure 13. Complete Interference in the SKINTEX Model

Test Material	Type of Interference	Solution
Intensely Colored Samples	Blanking problems	Activated diluent
Fluorescent Samples	Blanking problems	Activated diluent
Alkaline Samples	Decreasing curves	AMA protocol
Surfactants	Decreasing curves	5-volume procedure RMA

Table 2. Materials which can Exhibit Interference

EVALUATION OF COSMETIC COMPOUNDS

A study of 128 cosmetic formulations was performed using the SKINTEX UMA and AMA protocols. This study included materials from 14 product classes as described in Table 3.

Product Classes	Number of Samples
Perfumes/Colognes	7
Skin Cleanser	19
Raw Materials	12
Eyeliner	2
Eye Shadow	5
Hair Conditioners	17
Shampoo	8
OTC	32
Bath Preparations	1
Liquid Soaps	5
Foundation	9
Hand Cream	4
Face Powders	3
Moisturizers	9

Table 3. Product Classes Analyzed in the SKINTEX UMA and AMA

The number of irritants with *in vivo* scores of ≥ 3 in the Draize Dermal Irritation assay with a 24-hour occluded application on nonabraded skin was 33. Three irritants were underestimated which included a bath preparation, a liquid soap and a raw surfactant. Two compounds, sorbitol and xylitol produce false positive responses in SKINTEX(SEE Table 4.). Formulations with these materials were excluded from analysis. Six nonirritants including a liquid soap, two hair conditioners and two moisturizers were overestimated. In this study the specificity is 93% and the sensitivity is 90%. The product class of liquid soaps demonstrated problems of both over and under estimation. the correlation coefficient for the SKINTEX PDII to the *in vivo* PDII was $r=0.81$.

EVALUATION OF PURE CHEMICALS AND HOUSEHOLD PRODUCTS

Fifty-six pure chemicals were analyzed by the SKINTEX SCL method. The chemicals studied included acids, bases, solvents, preservatives, surfactants and dyes. Two acids with pH of 3 were overestimated in this study. Two solvents also produced results in the SKINTEX SCL which were greater than the *in vivo* results. The *in vivo* results were determined using a 4-hour occluded application with nonabrasion on the rabbit. The specificity was 85% due to this overestimation. Sensitivity was 96% demonstrating a good ability of the assay to screen irritants with two acids and two solvents.

A variety of household products were analyzed in the SKINTEX SCL. *In vivo* results for four occluded applications on nonabraded rabbits were compared to the results obtained by the SKINTEX SCL method. In this study of 30 formulations including shampoos, conditioners, cleansers, carpet, furniture cleansers and fresheners, there were 18 irritants produced and *in vivo* score greater than two with a 4-hour application.

Two formulations which produced higher results in the *in vitro* test than in the *in vivo* test were cleaners. A shampoo produced an *in vitro* PDII equivalent of 1.65 and the *in vivo* PDII score was 2.23. This material was classified as an irritant *in vivo* and a nonirritant *in vitro*. A gel with high fragrance content and an *in vivo* score of 3.2 was classified as a nonirritant with an *in vitro* PDII equivalent of 1.7.

EVALUATION OF HUMAN DERMAL IRRITANTS

Preliminary studies with the SKINTEX HRA method demonstrated the relevance of the SKINTEX model to predicting the *in vivo* endpoint in humans. If an *in vivo* score of greater than 1.2 is considered an irritant then SLS(2%), BAC(1%), phenols(12%, 15% and 20%) and HCL(10% and 20%) were classified as irritants in SKINTEX HRA and *in vivo* (see Figure 14, 15 and 16). With vehicles, surfactants, acids and other miscellaneous chemicals, the *in vitro* method produced results which correlated to the human results. For 50 diverse chemicals two test samples were underestimated and four test materials were over-estimated.

Sorbitol > 4%
Xylitol > 4%
Atricaud > 1%
Zn compounds > 1%

Table 4. Materials which Produce False Positive Results

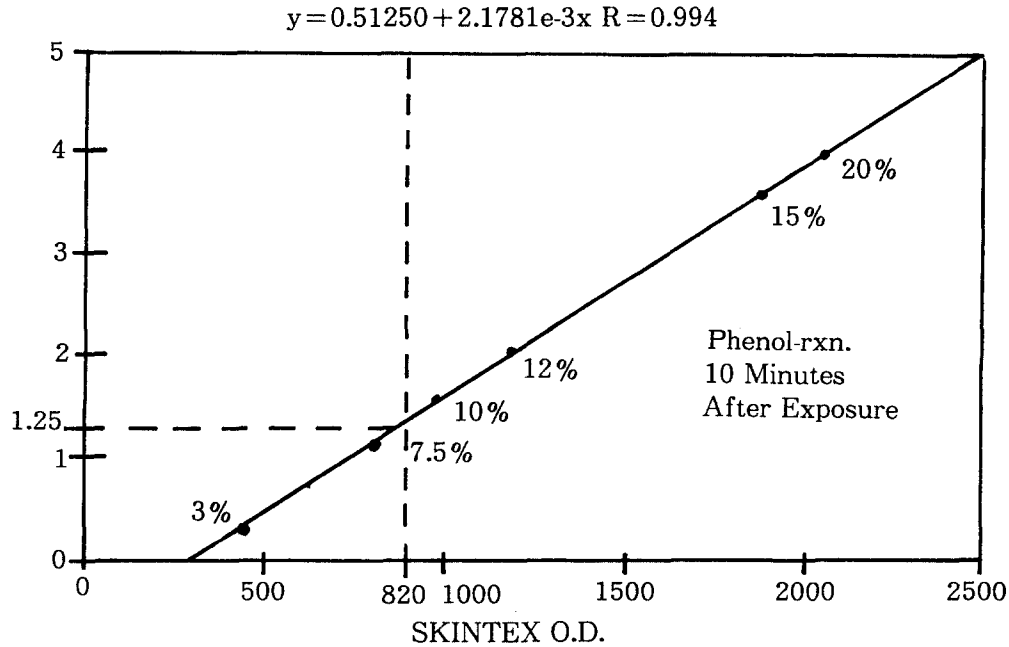

Figure 14. Human *In Vivo* Data vs. SKINTEX O.D.

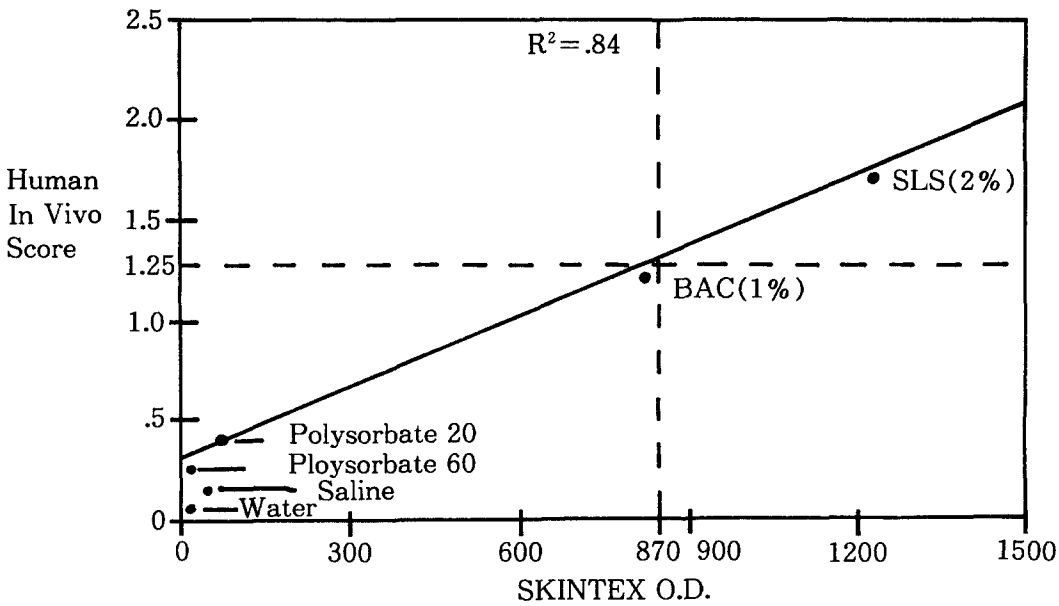

Figure 15. Human *In Vivo* Data vs. SKINTEX O.D.

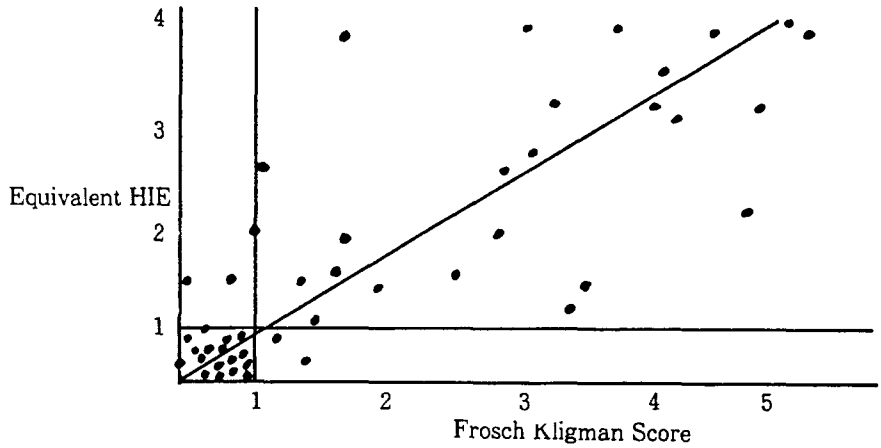

Figure 16. Human *In Vivo* vs. *In Vitro*

Two test materials which were overestimated had a pH of 3. The specificity of this study was 83%. The sensitivity was 92%. This expanded study demonstrates for diverse chemicals with varying mechanisms of toxicity the SKINTEX HRA predicts the *in vivo* human response (see Figure 16).

DISCUSSION

Evaluation of the SKINTEX System in studies of Cosmetics, Household Products and Chemicals was undertaken. New protocols specific to surfactants and alkaline materials were utilized. Evaluation of new protocols to predict *in vivo* human response and 4-hour *in vivo* application times in rabbits was also undertaken.

The capability of these protocols to provide a component of a test battery for dermal irritation has been shown. No one test will include the diverse mechanism and path ways of importance in the development of dermal irritation. The efficient use of methods with different capabilities, compatibilities and mechanisms can provide important information with respect to human safety.

A tier approach has been recommended to utilize *in virto* methods as screens or first stage of the tier testing. An example is presented in Figure 17.

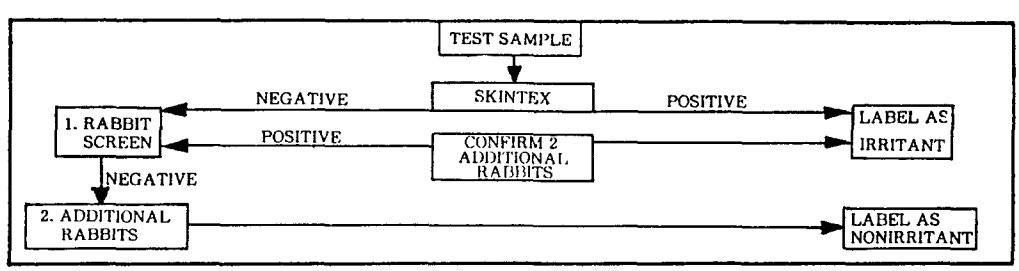

Figure 17. Approach to Testing

A good description of the important scientific basis, capabilities and limitations of the SKINTEX Approach and Cell Culture Approaches was presented by Dr. Michael Balls (14). This description is summarized in Figure 18.

THE EYTEX/SKINTEX APPROACH	THE CELL CULTURE APPROACH
Narrow mechanistic basis	Wide mechanistic basis
Tight Protocols in terms of dosage, exposure, etc.	Infinitely variable protocols in terms of dosage, exposure, etc.
Strong links to regulatory animal tests	Weak links to regulatory animal tests
Results readily classified to match regulatory classifications	Results not readily classlfied to match regulatory classifications
Able to handle awkward test materials	Not readily able to accommodate awkward test materials
Relatively narrow range of values obtainable	Relatively wide range of values obtained
Not suitable for repeat-dose studies	Suitable for repeat-dose studies
Not suitable for recovery studies	Suitable for recovery studies

Figure 18. A comparison of the EYTEX/SKINTEX Approach and the Cell Culture Approach

SUMMARY

The SKINTEX System includes several new protocols to more accurately analyze alkaline and surfactant materials and to predict different *in vivo* endpoints. A series of three major studies demonstrates the relevance of these protocols to the endpoint of dermal irritation at two application times in rabbits and man.

The SKINTEX System can provide a valuable component in a test battery. The use of target Biomacromolecules and cell cytotoxicity test combines the different capabilities and limitations of these methods effectively.

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