

ETAD



FYI-0797-1293

Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers
U.S. DYE MANUFACTURERS OPERATING COMMITTEE OF ETAD

July 8, 1997

Dr. Leonard Keifer
Risk Assessment Division
MC 7403
U.S. Environmental Protection Agency
401 M Street, SW
Washington, DC 20460

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Dear Dr. Keifer:

As discussed on the telephone on June 24, I am enclosing for your information copies of three final reports for ETAD studies on the in vitro skin penetration of certain dyes. The dyes studied include C. I. Disperse Yellow 3, C. I. Disperse Red 17, C. I. Disperse Red 60, C. I. Disperse Yellow 64, C. I. Disperse Orange, C. I. Disperse Blue 165, C. I. Disperse Blue 79:1, C. I. Reactive Blue 19, and C. I. Direct Blue 218.

These studies were conducted in an effort to supplement existing hazard assessments and to provide a better basis for exposure assessment. Despite certain technical limitations in the performance of these studies, the results were conclusive concerning relative rates of skin penetration and demonstrated that only a very small fraction of the applied dye penetrates the skin barrier.

Please let me know if you have any comments or questions about these studies. You may phone me directly at 202-414-4154 or fax at 202-289-8584.

Sincerely,

C. Tucker Helmes, Ph.D.
Executive Director

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Enclosures

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Y08537/001, Y08538/001
Y08553/001, Y08558/001
CTL Study No: JV1436
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REPORT NO: CTL/L/5926

IN VITRO ABSORPTION OF VARIOUS DYES THROUGH
HUMAN AND PIG EPIDERMIS

CIRCULATION LIST

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- 1 Report Centre Reference Copy
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- 3 Dr I F H Purchase/Dr J E Doe/Dr E D Brown
- 4 Dr J W Botham
- 5 Mr R J Ward
- 6 Mr B H Woolfen

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- 7 Dr G Green-Buckley, Zeneca Specialties, Blackley
- 8 Dr J R Easton, Zeneca Specialties, Blackley
- 9-20 Dr H Motschi, ETAD, Clarastrasse 4, CH-4005, Basel 5, Switzerland
(for ETAD Technical Committee Members)

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IN VITRO ABSORPTION OF VARIOUS DYES THROUGH
HUMAN AND PIG EPIDERMIS

by

R J Ward

THE DATA IN THIS REPORT HAVE NOT BEEN QUALITY ASSURED

Approved for Issue:

Dr J W Botham 
Product Toxicologist

Date of Issue: 20 DEC 1994

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IN VITRO ABSORPTION OF VARIOUS DYES
THROUGH HUMAN AND PIG EPIDERMIS

1. SUMMARY

The in vitro absorption of six disperse dyes (disperse red 60, disperse yellow 64, disperse red 17, disperse yellow 3, disperse blue 165 and T2030/76) has been measured through human and pig epidermis. The dyes were prepared as suspensions ($1000\mu\text{g ml}^{-1}$) in a 0.5% solution of TWEEN 80 in distilled water which were applied to the epidermal membranes at a rate of $200\mu\text{l cm}^{-2}$ ($\approx 200\mu\text{g cm}^{-2}$). Absorption was measured under occlusion during an exposure period of 55hr.

The most slowly absorbed dye was disperse blue 165 (maximum rates $<0.04\mu\text{g cm}^{-2}\text{ hr}^{-1}$ for human and $0.013\mu\text{g cm}^{-2}\text{ hr}^{-1}$ for pig). The fastest absorption rates were measured from disperse yellow 3 (maximum rates $0.219\mu\text{g cm}^{-2}\text{ hr}^{-1}$ for human and $2.59\mu\text{g cm}^{-2}\text{ hr}^{-1}$ for pig). For all the dyes, absorption through pig epidermis over predicted absorption through human epidermis by factors of up to 17.1 (disperse red 17).

2. INTRODUCTION

The in vitro absorption of various dyes been measured to obtain information on their potential to be absorbed through human and pig epidermis. Six disperse dyes were tested:

Disperse Red 60
Disperse Yellow 64
Disperse Red 17
Disperse Yellow 3
Disperse Blue 165
T2030/76

Absorption was measured using glass diffusion cells (Figure 1) employing established methodology (Scott and Clowes, 1992).

2. METHOD

Extraneous tissue was removed from human whole skin samples and pig whole skin was separated from the cartilage of pig ears. The whole skin samples from both species were immersed in water at 60°C for 40-50 seconds. The epidermis was gently teased off the dermis and stored deep frozen on aluminium foil until required for use.

Samples of human or pig epidermis were mounted in glass diffusion cells and the integrity of the membranes determined by measurement of their permeability to tritiated water. Membranes displaying a permeability coefficient of $<1.5 \times 10^{-3} \text{cm hr}^{-1}$ (human) or $<4.5 \times 10^{-3} \text{cm hr}^{-1}$ (pig) were regarded as being undamaged and used for exposure to the test dyes. Each dye was mixed with a solution of 0.5% TWEEN 80 in distilled water to give a dye concentration of $1000 \mu\text{g ml}^{-1}$ and at this concentration all the dyes remained in suspension. The suspensions were ultra-sonicated to disperse any large particles and were applied to the epidermal membranes immediately after preparation to ensure maximum homogeneity. The application rate was $200 \mu\text{l cm}^{-2}$ (equivalent to $20 \mu\text{g dye cm}^{-2}$). The donor chambers were occluded to prevent any evaporation of the vehicle during the exposure period (55hr). At recorded intervals throughout the exposure period, samples (0.5ml) of the receptor fluid (50% ethanol in distilled water) were taken from the receptor chamber for analysis. The volume of fluid in the receptor chamber was maintained by the addition of 0.5ml of fresh receptor fluid to the chamber immediately after the removal of each sample.

The samples taken during the exposure period were analysed by high performance liquid chromatography (HPLC), with the limits of determination varying between 0.02 and $0.05 \mu\text{g ml}^{-1}$, dependent upon the dye. The results of the analyses were used to calculate the amount ($\mu\text{g cm}^{-2}$) of dye absorbed

at each sample time point and to determine the absorption profiles ($\mu\text{g cm}^{-2}$ v time). The absorption rates ($\mu\text{g cm}^{-2} \text{ hr}^{-1}$) were calculated from the slope of the profiles between a chosen range of time points. The maximum absorption rates is represented by the linear portion of the profile.

3. RESULTS AND DISCUSSION

The mean absorption profiles for each dye through human and pig epidermis are displayed in Figures 2-7 and the mean absorption data in Table 1. The data given in Table 1 represent the absorption rate, if measurable, during a working day period (1-10hr) and the maximum rate determined for each dye. Where absorption was below the limit of determination, values equivalent to the limit of determination have been used in calculations for the mean absorption rates.

The most poorly absorbed of the dyes was Disperse Blue 165, from which no absorption was detected through human epidermis ($<0.004\mu\text{g cm}^{-2} \text{ hr}^{-1}$) and from pig epidermis absorption was only detected 48hr after exposure through 3 of the 6 membranes used ($0.013\mu\text{g cm}^{-2} \text{ hr}^{-1}$). From the remaining dyes, absorption was detected through both human and pig epidermis within the first 10hr after application. The fastest absorption rates were measured from Disperse Yellow 3 (human = $0.219\mu\text{g cm}^{-2} \text{ hr}^{-1}$; pig = $2.59\mu\text{g cm}^{-2} \text{ hr}^{-1}$) during the latter maximum periods of absorption.

For all 6 dyes absorption rates were faster through pig epidermis than through human epidermis. With the exception of Disperse Blue 165, where the difference between pig and human epidermis could only be calculated as >3.25 , the factors of difference (pig $>$ human) during the latter period varied between 6.1 for Disperse Yellow 64 and 17.1 for Disperse Red 17.

The absorption rates obtained for the dyes in this study were very slow when compared with the absorption rate of other penetrants measured using this technique (Dugard and Scott, 1984; Dugard et al, 1984). The ranking order for maximum absorption rates was the same for human and pig skin (Figure 7). The results indicate that, although pig skin over-predicts absorption through human skin in vitro test system, it can be used to predict the relative rates of absorption of disperse dyes through human skin.

.....
R J Ward

R J Ward
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(Study Director)

9th Dec 84
.....
Date

4. REFERENCES

Dugard P H and Scott R C (1984). Absorption Through Skin. The Chemotherapy of Psoriasis, International Encyclopedia of Pharmacology and Therapeutics; Section 110, Pergamon Press, Oxford and New York, pp.125-144

Dugard P H, Walker M, Mawdsley S J and Scott R C (1984). Absorption of Some Glycol Ethers Through Human Skin In Vitro. Environ. Health Persp. 57 193-197.

Scott R C and Clowes H M (1992). In Vitro Percutaneous Absorption Experiments: A Guide to the Techniques for Use in Toxicology Assessments. Toxicology Methods, Vol. 2, No. 2, pp.113-123.

IN VITRO ABSORPTION OF VARIOUS DYES
THROUGH HUMAN AND PIG EPIDERMIS

TABLE 1

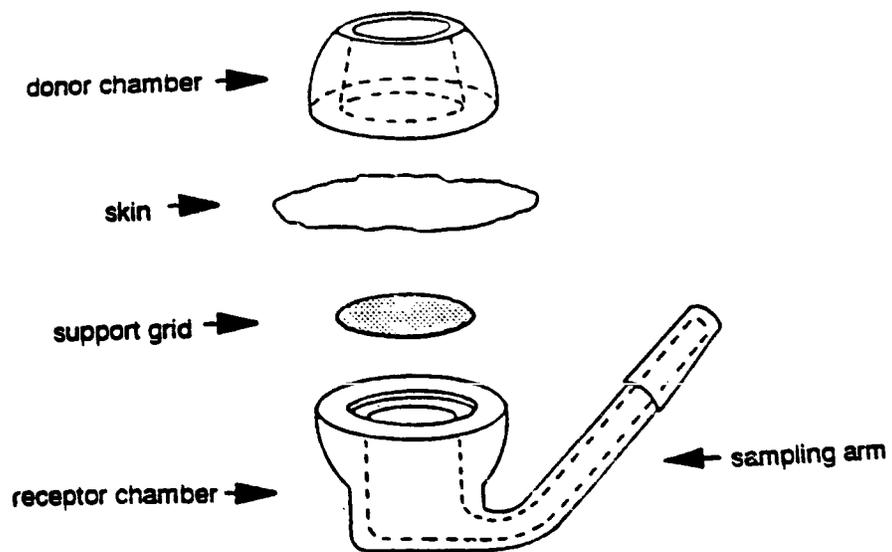
SUMMARY OF THE MEAN ABSORPTION RATES THROUGH
HUMAN AND PIG EPIDERMIS

Dye	Mean Absorption Rates (n=6)			
	Human Epidermis		Pig Epidermis	
	Time Period (hr)	$\mu\text{g cm}^{-2} \text{ hr}^{-1} \pm \text{SEM}$	Time Period (hr)	$\mu\text{g cm}^{-2} \text{ hr}^{-1} \pm \text{SEM}$
Disperse Red 60	1-10	0.013 ± 0.002	1-10	0.068 ± 0.005
	4-55	0.020 ± 0.004	10-55	0.207 ± 0.023
Disperse Yellow 64	1-10	0.007 ± 0.000	1-10	0.030 ± 0.003
	10-55	$0.008 \pm <0.001$	4-55	0.049 ± 0.004
Disperse Red 17	1-10	0.068 ± 0.009	1-10	0.158 ± 0.005
	4-30	0.105 ± 0.014	31-55	1.80 ± 0.278
Disperse Yellow 3	1-10	0.163 ± 0.035	1-10	0.508 ± 0.050
	6-55	0.219 ± 0.057	31-55	2.59 ± 0.233
Disperse Blue 165	1-10	<0.007	1-10	<0.007
	1-55	<0.001	31-55	0.013 ± 0.006
T2030/76	01-10	<0.003	1-10	0.012 ± 0.001
	10-55	0.004 ± 0.001	24-55	0.050 ± 0.007

Where absorption was below the limit of determination LOD, the value for the LOD has been included to calculate the mean.

IN VITRO ABSORPTION OF VARIOUS DYES
THROUGH HUMAN AND PIG EPIDERMIS

FIGURE 1
DIFFUSION CELL

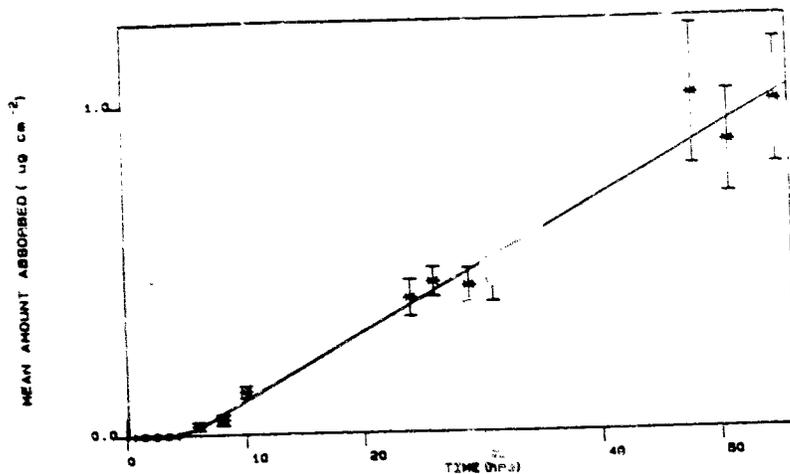


IN VITRO ABSORPTION OF VARIOUS DYES
THROUGH HUMAN AND PIG EPIDERMIS

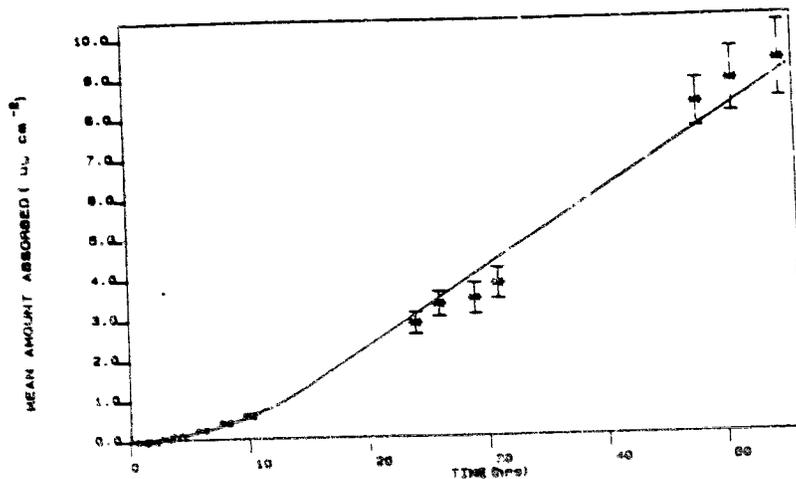
FIGURE 2

MEAN ABSORPTION PROFILES FOR
DISPERSE RED 60

a. Human Epidermis (n=6)



b. Pig Epidermis (n=6)

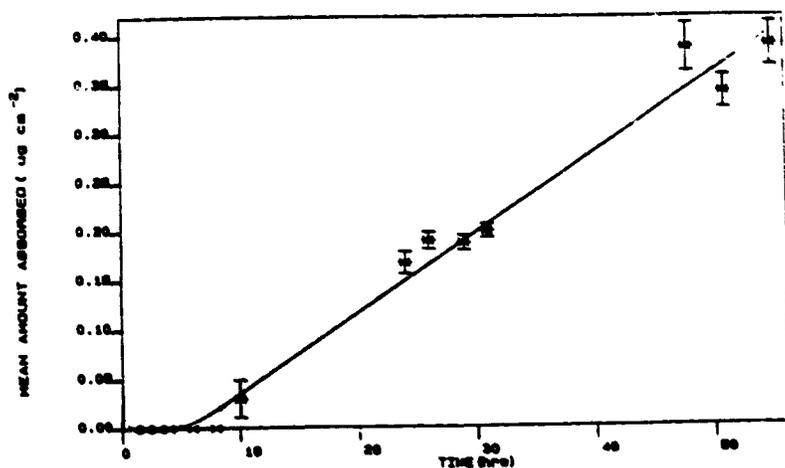


IN VITRO ABSORPTION OF VARIOUS DYES
THROUGH HUMAN AND PIG EPIDERMIS

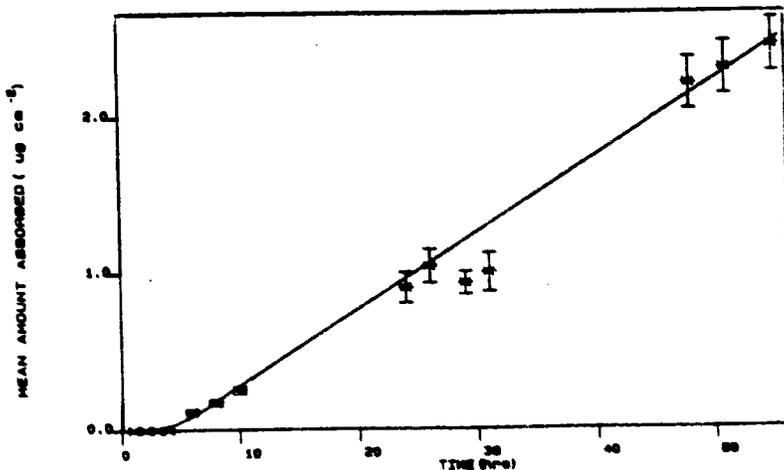
FIGURE 3

MEAN ABSORPTION PROFILES FOR
DISPERSE YELLOW 64

a. Human Epidermis (n=6)



b. Pig Epidermis (n=6)

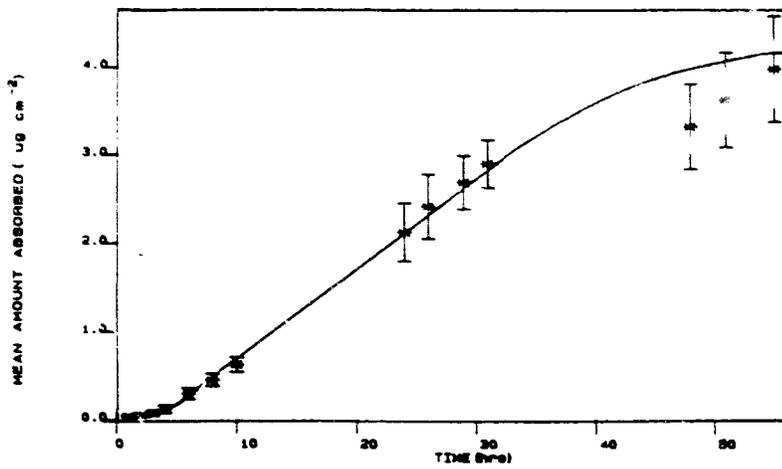


IN VITRO ABSORPTION OF VARIOUS DYES
THROUGH HUMAN AND PIG EPIDERMIS

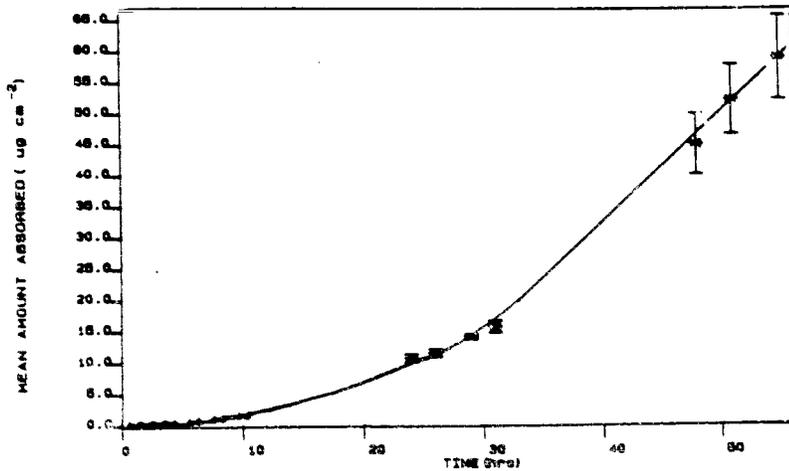
FIGURE 1

MEAN ABSORPTION PROFILES FOR
DISPERSE RED 17

a. Human Epidermis (n=6)



b. Pig Epidermis (n=6)

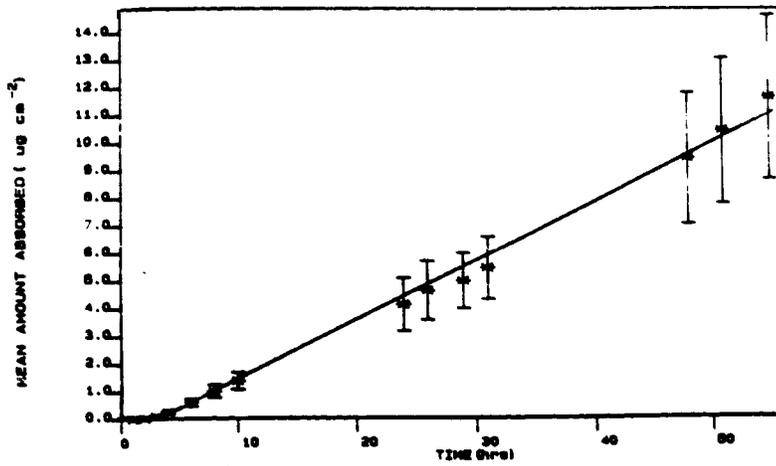


IN VITRO ABSORPTION OF VARIOUS DYES
THROUGH HUMAN AND PIG EPIDERMIS

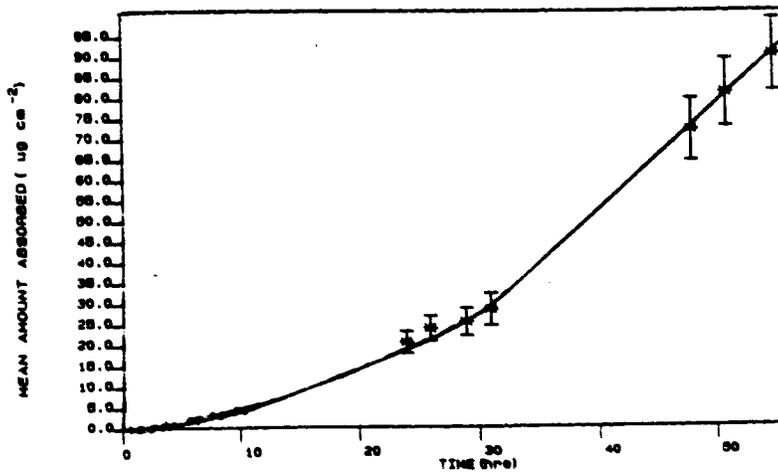
FIGURE 5

MEAN ABSORPTION PROFILES FOR
DISPERSE YELLOW 3

a. Human Epidermis (n=6)



b. Pig Epidermis (n=6)



IN VITRO ABSORPTION OF VARIOUS DYES
THROUGH HUMAN AND PIG EPIDERMIS

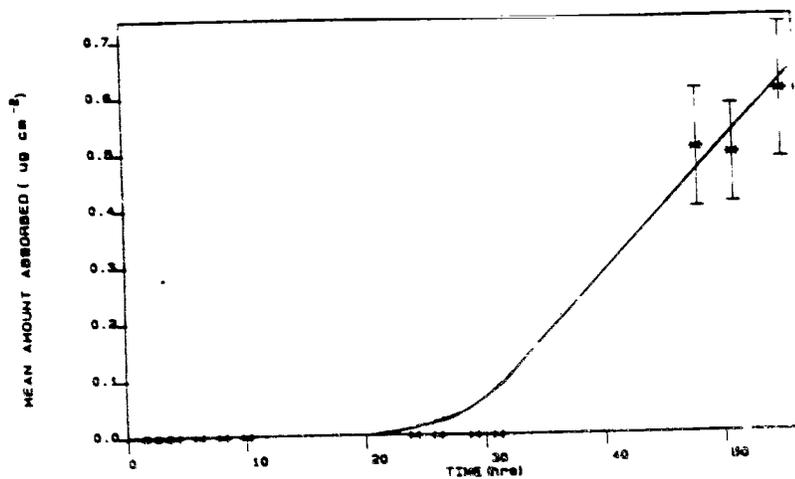
FIGURE 6

MEAN ABSORPTION PROFILES FOR
DISPERSE BLUE 165

a. Human Epidermis

NO ABSORPTION DETECTED

b. Pig Epidermis (n=3)

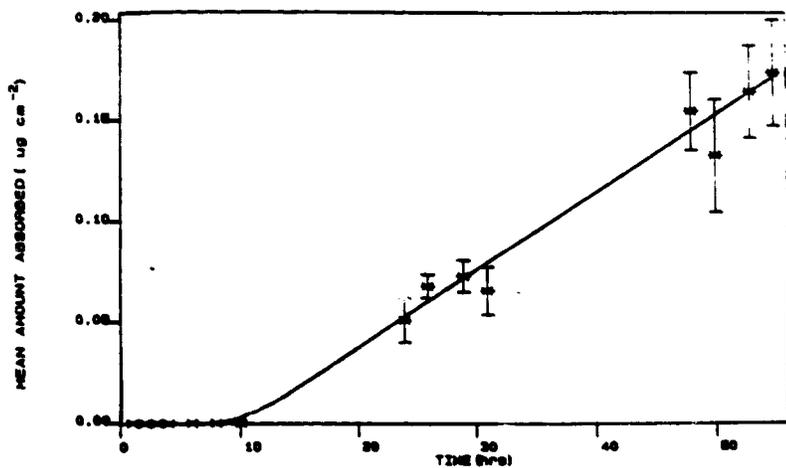


IN VITRO ABSORPTION OF VARIOUS DYES
THROUGH HUMAN AND PIG EPIDERMIS

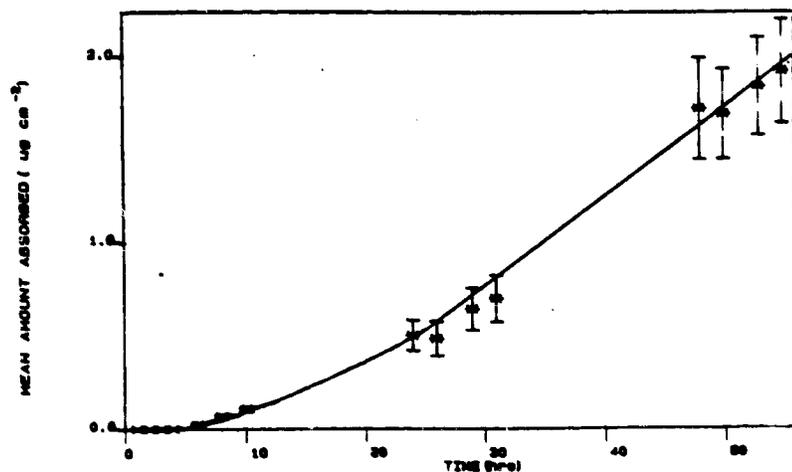
FIGURE 7

MEAN ABSORPTION PROFILES FOR
T2030/76

a. Human Epidermis (n=6)



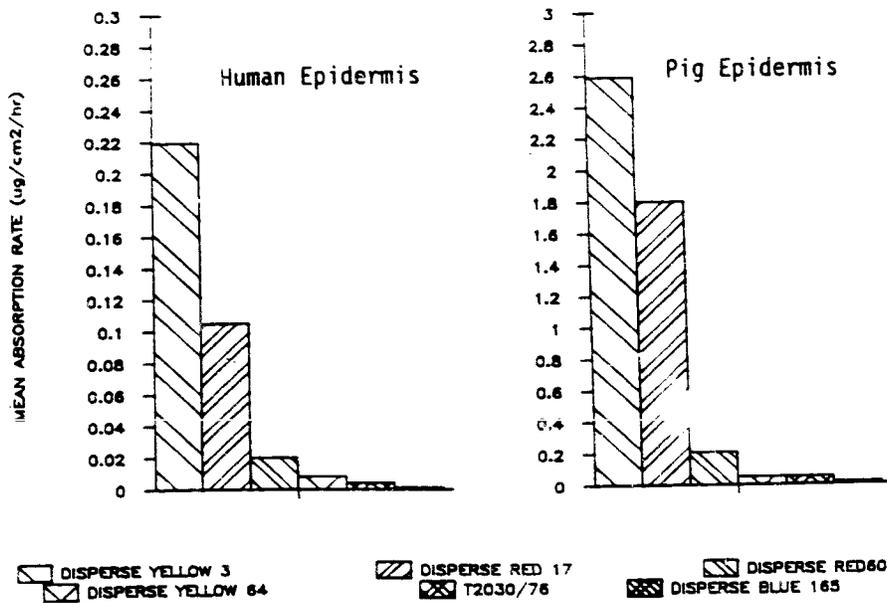
b. Pig Epidermis (n=6)



IN VITRO ABSORPTION OF VARIOUS DYES
THROUGH HUMAN AND PIG EPIDERMIS

FIGURE 8

COMPARISON OF MAXIMUM ABSORPTION RATES
FOR EACH DYE



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REPORT NO: CTL/L/6841

DISPERSE RED 60 AND DISPERSE YELLOW 3:
IN VITRO ABSORPTION FROM SYNTHETIC PERSPIRATION
AND FIVE FORMULATIONS THROUGH HUMAN AND PIG EPIDERMIS

CIRCULATION LIST

Internal

- 1 Report Centre Reference Copy
- 2 Report Centre - Spare
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- 8 Dr J R Easton, Zeneca Specialties Blackley
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by

R J Ward

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Approved for Issue:

M. Padgham
M D J Padgham
Contract Manager

Date of Issue: 6 DEC 1995

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DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATIONS
THROUGH HUMAN AND PIG EPIDERMIS

1. SUMMARY

The in vitro absorption of the disperse dyes red 60 and yellow 3 has been measured from saturated solutions in synthetic perspiration and from five formulations of each dye. The formulations contained 2.5% dye and 7.5% of one of five dispersing agents (Sodium Dispersol, Ultrazine NA, Ufoxane 2, Reax 85A or Dynasperse A). Absorption was measured through human epidermis and, for one formulation of disperse yellow 3, through pig epidermis.

The solutions of dye in synthetic perspiration were applied at $10\mu\text{l cm}^{-2}$ and as bulk applications of $200\mu\text{l cm}^{-2}$. The dye formulations were applied only at $10\mu\text{l cm}^{-2}$. The $10\mu\text{l cm}^{-2}$ applications, which were designed to simulate potential dermal exposure, were left unoccluded for the duration of the exposure period (55hr), while the higher applications were occluded.

For the applications to human skin in synthetic perspiration at a rate of $10\mu\text{l cm}^{-2}$, no absorption of either dye was detected ($<0.001\mu\text{g cm}^{-2}\text{ hr}^{-1}$). At $200\mu\text{l cm}^{-2}$ the respective absorption rates for disperse yellow 3 and disperse red 60 were 0.09 and $0.02\mu\text{g cm}^{-2}\text{ hr}^{-1}$. These results indicate that the absorption of these dyes from perspiration is likely to be very low.

Absorption of the formulated dyes through human epidermis was slow. For the disperse red 60 formulations, absorption rates for the dye ranged from $0.005\mu\text{g cm}^{-2}\text{ hr}^{-1}$ (Ultrazine NA and Ufoxane 2 formulations) to $<0.001\mu\text{g cm}^{-2}\text{ hr}^{-1}$ (Reax 85A formulation). From the disperse yellow 3 formulations the range was $0.012\mu\text{g cm}^{-2}\text{ hr}^{-1}$ (sodium dispersol formulation) to $<0.001\mu\text{g cm}^{-2}\text{ hr}^{-1}$ (Reax 85A and Dynasperse A formulations).

Absorption of disperse yellow 3 through pig epidermis from the Reax 85A formulation (up to $0.122\mu\text{g cm}^{-2}\text{ hr}^{-1}$) was at least 100 times faster than than through human epidermis.

These results indicate that the absorption of these dyes, although very slow, was affected by the presence of the dispersing agents and that their effect on absorption rate was not necessarily the same for each of the two dyes tested.

2. INTRODUCTION

The in vitro percutaneous absorption of the disperse dyes red 60 and yellow 3 have been measure from solution in a synthetic perspiration and from formulations containing different dispersing agents.

Absorption was measured using glass diffusion cells (Figure 1) employing established methodology (Scott and Clowes, 1992).

3. METHOD

3.1 Preparation of Epidermal Membranes

Extraneous tissue was removed from human whole skin samples and pig whole skin was separated from the cartilage of pig ears. The whole skin samples from both species were immersed in water at 60°C for 40-50 seconds. The epidermis was gently teased away from the dermis and stored deep frozen on aluminium foil until required for use.

3.2 Assembly of Diffusion Cell and Assessment of Membrane Integrity.

Samples of human or pig epidermis were mounted in glass diffusion cells and the integrity of the membranes determined by measurement of their permeability to tritiated water. Membranes displaying a permeability

coefficient of $<1.5 \times 10^{-3} \text{cm hr}^{-1}$ (human) or $<4.5 \times 10^{-3} \text{cm hr}^{-1}$ (pig) were regarded as being undamaged and used for exposure to the test dyes.

3.3 Determination of the Absorption of the Test Dyes from Synthetic Perspiration.

The synthetic perspiration solution was prepared as described in BS1009: 1990 Section E04; Paragraph 4.4 (acid solution). The solution was freshly prepared before use and contained per litre of water:

- 0.5g 1-histidine monohydrochloride monohydrate
- 0.5g sodium chloride
- 2.2g sodium dihydrogen orthophosphate dihydrate

The resulting solution was adjusted to pH5.5 by the addition of 0.1M sodium hydroxide.

Aliquots of the standard material for each dye were mixed on a magnetic stirrer with the synthetic perspiration solution for 1hr at room temperature to achieve saturated solutions. The resulting mixtures were centrifuged and the supernatants sampled for analysis prior to dosing.

Each supernatant solution was applied to the epidermal membranes at a rate of $10 \mu\text{l cm}^{-2}$ and $200 \mu\text{l cm}^{-2}$, with the higher dose being occluded for the duration of the exposure period (55hr). At recorded intervals throughout the exposure period, samples (0.5ml) of the receptor fluid (50% v/v ethanol in distilled water) were taken from the receptor chamber for analysis. The volume of fluid in the receptor chamber was maintained by the addition of 0.5ml of fresh receptor fluid to the chamber immediately after the removal of each sample.

The samples taken during the exposure period were analysed by high performance liquid chromatography (HPLC). The limits of determination were $0.03 \mu\text{g ml}^{-1}$ for disperse red 60 and $0.04 \mu\text{g ml}^{-1}$ for disperse yellow 3. Where absorption of the dyes was detected, the results of the analyses were used to calculate the amount ($\mu\text{g cm}^{-2}$) of dye absorbed at each sample time

point and to determine the absorption profiles ($\mu\text{g cm}^{-2}$ v time). The absorption rates ($\mu\text{g cm}^{-2} \text{ hr}^{-1}$) were calculated from the slope of the profiles between a chosen range of time points.

3.4 Determination of the Absorption of the Test Dyes from Formulations.

Five test formulations per dye were studied. The formulations contained 2.5% of dye and one of five dispersing agents (sodium dispersol, Ultrazine NA, Ufoxane 2, Reax 85A or Dynaspense A) at a concentration of 7.5%.

The test formulations were applied directly to epidermal membranes at a rate of $10\mu\text{l cm}^{-2}$ and left unoccluded for the duration of the exposure period. The receptor solution, sampling and analysis were as described in Section 3.3.

4. RESULTS AND DISCUSSION

Where absorption of the dyes was detected, the mean absorption profiles are displayed in Figures 2-7. The mean absorption rate data are given in Tables 1-3. Where absorption was below the limit of quantitation ($0.001\mu\text{g cm}^{-2} \text{ hr}^{-1}$ for both dyes), values equivalent to the limit of quantitation have been used in calculations to obtain the mean absorption rates.

4.1 Absorption from Artificial Perspiration.

Analysis of the supernatant saturated dye solutions in synthetic perspiration gave concentrations of $24.4\mu\text{g ml}^{-1}$ for disperse red 60 and $20.9\mu\text{g ml}^{-1}$ for disperse yellow 3.

No absorption ($<0.001\mu\text{g cm}^{-2} \text{ hr}^{-1}$) of either dye was detected from the $10\mu\text{l cm}^{-2}$ applications of these saturated solutions. From the $200\mu\text{l cm}^{-2}$ occluded application of disperse red 60 (Table 1), absorption was not

detected until after 10 hours after application (Figure 2), after which time an absorption rate of $0.020\mu\text{g cm}^{-2}\text{ hr}^{-1}$ was achieved. From the $200\mu\text{l cm}^{-2}$ application of disperse yellow 3 (Table 1) absorption was measurable after 2 hours (Figure 3) at a rate of $0.090\mu\text{g cm}^{-2}\text{ hr}^{-1}$.

The mean absorption rate obtained for the disperse red 60 applied at $200\mu\text{l cm}^{-2}$ was the same as that obtained from an earlier study (Report No CTL/L/5926), when the dyes were applied at $1000\mu\text{g ml}^{-1}$ in water containing 0.5% TWEEN 80. The mean rate obtained for disperse yellow 3 was approximately twice the rate obtained in the earlier study.

4.2 Absorption from Formulations

No absorption ($<0.001\mu\text{g cm}^{-2}\text{ hr}^{-1} = 0.02\%$ of applied dose at 55hr) of disperse red 60 (Table 2) was detected from the Reax 85 formulation or of disperse yellow 3 (Table 3) from either the Reax 85 or Dynasperse A through human epidermis. The absorption rate for disperse red 60 from Dynasperse A was $0.003\mu\text{g cm}^{-2}\text{ hr}^{-1}$ (0.07% of applied dose at 55hr).

Mean absorption of both dyes from the Ultrazine NA and Ufoxane 2 formulations were the same ($0.006\mu\text{g cm}^{-2}\text{ hr}^{-1}$; 0.13% of applied dose at 55hr). The fastest absorption rates for disperse red 60 were from these Ultrazine NA and Ufoxane 2 formulations, but for disperse yellow 3, the fastest rate was from the Sodium Dispersol formulation ($0.012\mu\text{g cm}^{-2}\text{ hr}^{-1}$; 0.26% of applied dose at 55hr).

From the application of the Reax 85 formulation to pig epidermis the maximum absorption rate was measured between 1-24hr ($0.122\mu\text{g cm}^{-2}\text{ hr}^{-1}$). The average rate over 55hr was $0.087\mu\text{g cm}^{-2}\text{ hr}^{-1}$ (2% of applied dose at 55hr).

These results indicate that the absorption of these dyes, although very slow, was affected by the presence of the dispersing agents and that their effect on absorption rate was not necessarily the same for each of the two dyes tested.

5. REFERENCES

Scott R C and Clowes H M (1992). In Vitro Percutaneous Absorption Experiments: A Guide to the Techniques for Use in Toxicology Assessments. Toxicology Methods, Vol. 2, No. 2, pp.113-123.

.....
R J Ward (Study Director)

20th November '95
.....
Date

DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
 FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATION
 THROUGH HUMAN AND PIG EPIDERMIS

TABLE 1

ABSORPTION FROM SYNTHETIC PERSPIRATION

Dye	Application ($\mu\text{l cm}^{-2}$)	Time Period (hr)	Mean Absorption Rate ($\mu\text{g cm}^{-2} \text{ hr}^{-1} \pm \text{SEM}$)
Disperse Red 60	10	2-55	<0.001 (n=6)
	200	10-55	0.020 \pm 0.004 (n=6)
Disperse Yellow 3	10	2-55	<0.001 (n=5)
	200	2-55	0.090 \pm 0.005 (n=6)

DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
 FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATION
 THROUGH HUMAN AND PIG EPIDERMIS

TABLE 2

ABSORPTION OF DISPERSE RED 60 FROM FORMULATIONS
 THROUGH HUMAN EPIDERMIS

Dispersing Agent	Time Period (hrs)	Mean Absorption Rate ($\mu\text{g cm}^{-2} \text{ hr}^{-1} \pm \text{SEM}$)
Sodium Dispersol	31-55	0.002 \pm 0.001 (n=6)
Ultrazine NA	31-55	0.006 \pm 0.002 (n=6)
Ufoxane 2	24-55	0.006 \pm 0.002 (n=6)
Reax 85A	24-55	<0.001 (n=5)
Dynasperse A	2-55	0.003 \pm 0.001 (n=5)

DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
 FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATION
 THROUGH HUMAN AND PIG EPIDERMIS

TABLE 3

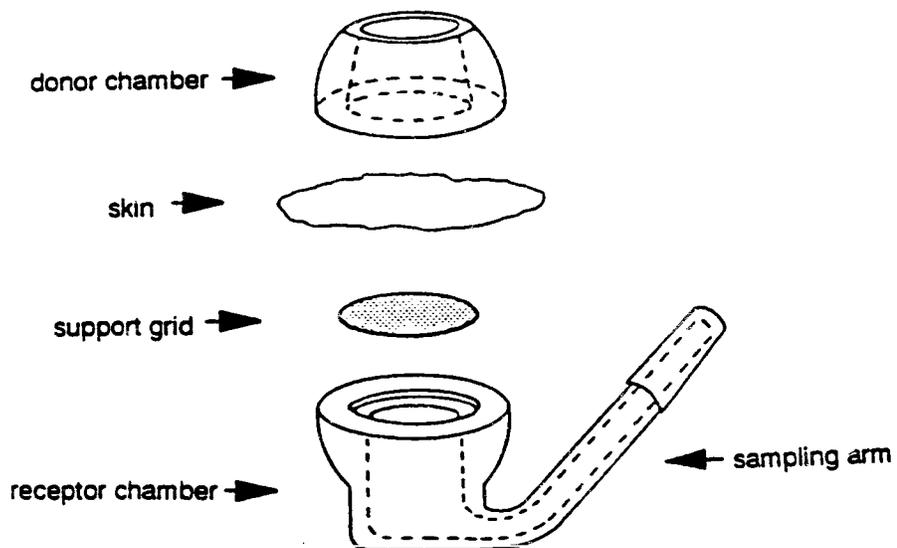
ABSORPTION OF DISPERSE YELLOW 3 FROM FORMUATIONS
 THROUGH HUMAN AND PIG EPIDERMIS

Dispersing Agent	Time Period (hrs)	Mean Absorption Rate ($\mu\text{g cm}^{-2} \text{ hr}^{-1} \pm \text{SEM}$)
Sodium Dispersol	6-55	0.012 \pm 0.005 (n=5)
Ultrazine NA	10-55	0.006 \pm 0.002 (n=6)
Ufoxane 2	10-55	0.006 \pm 0.003 (n=6)
Reax 85A Human	2-55	<0.001 (n=5)
Pig	1-24	0.122 \pm 0.039 (n=6)
	1-55	0.087 \pm 0.029 (n=6)
Gynasperse A	2-55	<0.001 (n=5)

DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATION
THROUGH HUMAN AND PIG EPIDERMIS

FIGURE 1

DIFFUSION CELL

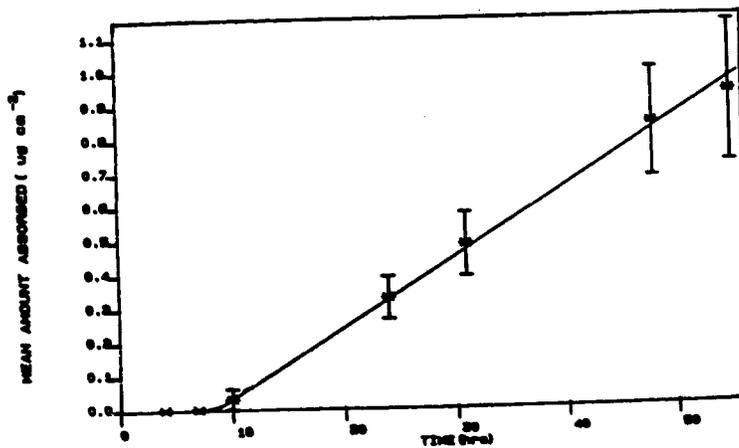


DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATION
THROUGH HUMAN AND PIG EPIDERMIS

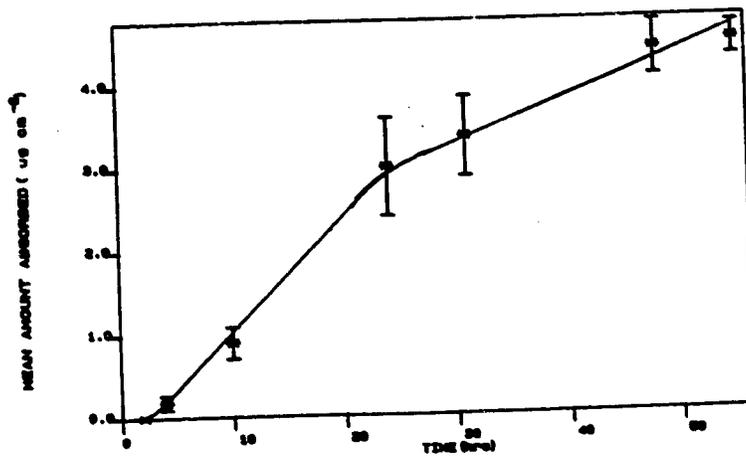
FIGURE 2

MEAN ABSORPTION PROFILES FOR
DISPERSE RED 60 AND DISPERSE YELLOW 3
FROM SYNTHETIC PERSPIRATION
(Application Rate $200\mu\text{l cm}^{-2}$)

a. Disperse Red 60 (n=6)



b. Disperse Yellow 3 (n=6)

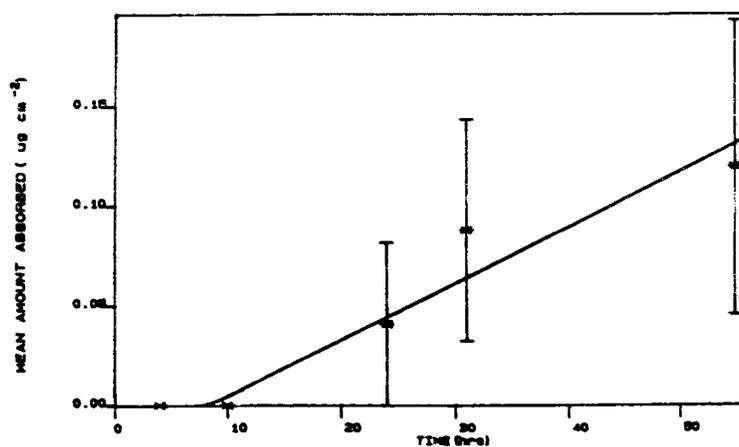


DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATION
THROUGH HUMAN AND PIG EPIDERMIS

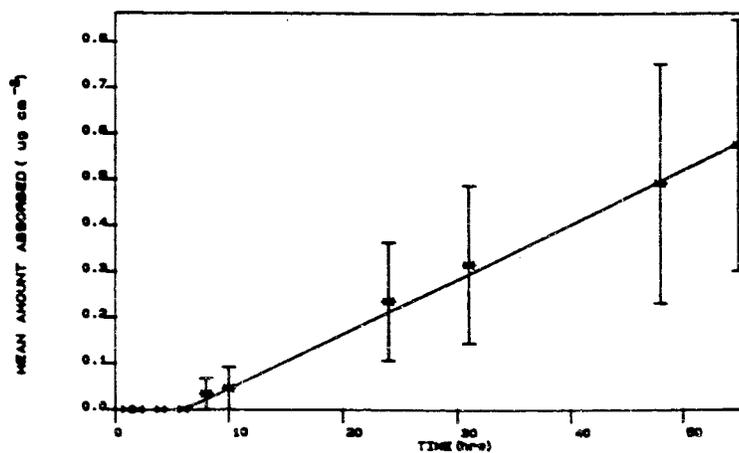
FIGURE 3

MEAN ABSORPTION PROFILES FOR
DISPERSE RED 60 AND DISPERSE YELLOW 3
FROM SODIUM DISPERSOL THROUGH HUMAN EPIDERMIS

a. Disperse Red 60 (n=6)



b. Disperse Yellow 3 (n=5)

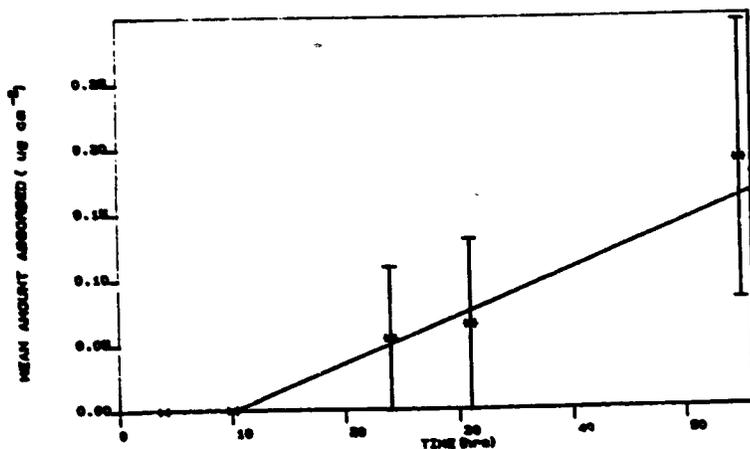


DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATION
THROUGH HUMAN AND PIG EPIDERMIS

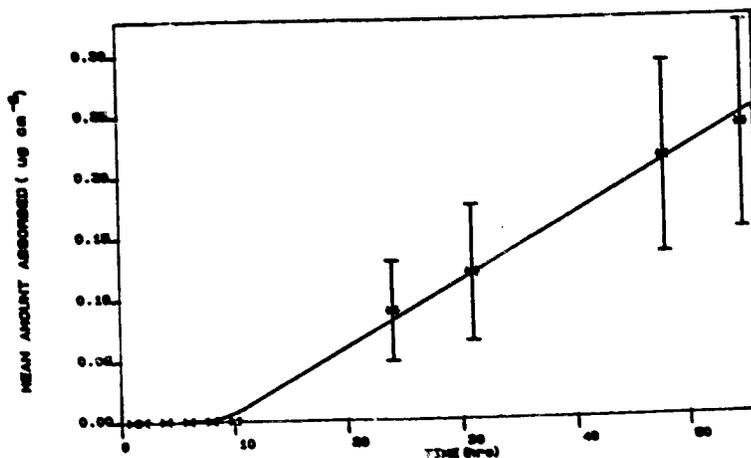
FIGURE 4

MEAN ABSORPTION PROFILES FOR
DISPERSE RED 60 AND DISPERSE YELLOW 3
FROM ULTRAZINE NA THROUGH HUMAN EPIDERMIS

a. Disperse Red 60 (n=6)



b. Disperse Yellow 3 (n=6)

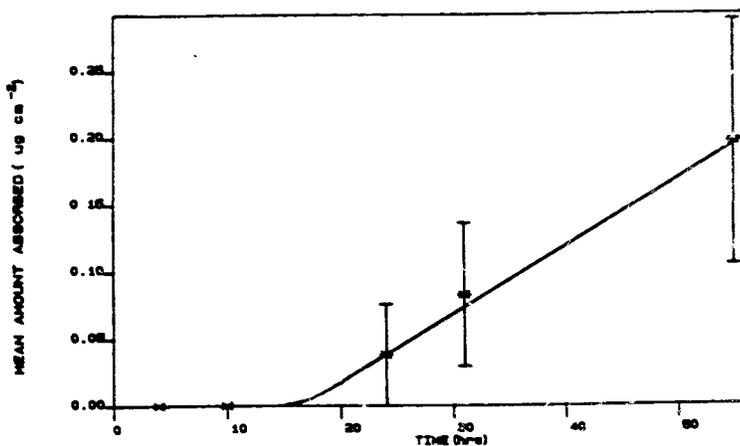


DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATION
THROUGH HUMAN AND PIG EPIDERMIS

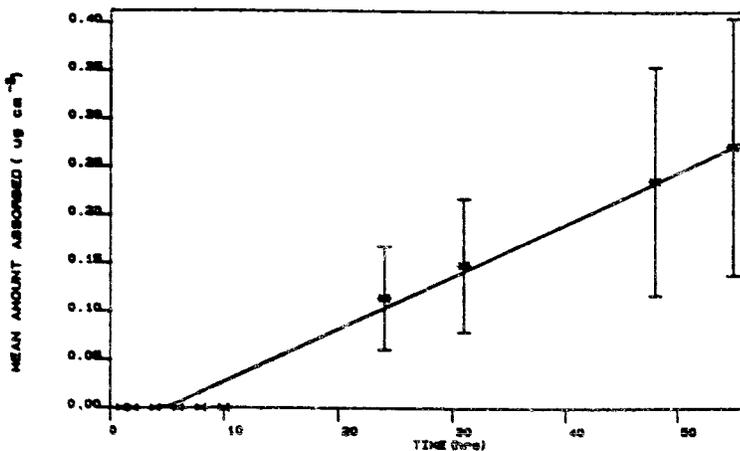
FIGURE 5

MEAN ABSORPTION PROFILES FOR
DISPERSE RED 60 AND DISPERSE YELLOW 3
FROM UFOXANE 2 THROUGH HUMAN EPIDERMIS

a. Disperse Red 60 (n=6)



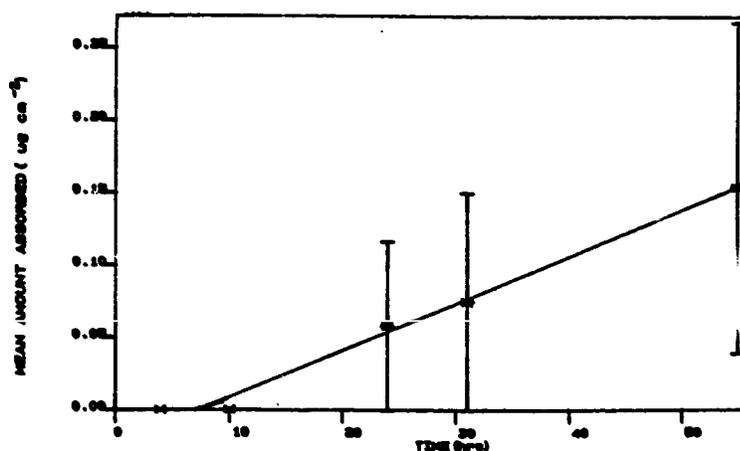
b. Disperse Yellow 3 (n=6)



DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATION
THROUGH HUMAN AND PIG EPIDERMIS

FIGURE 6

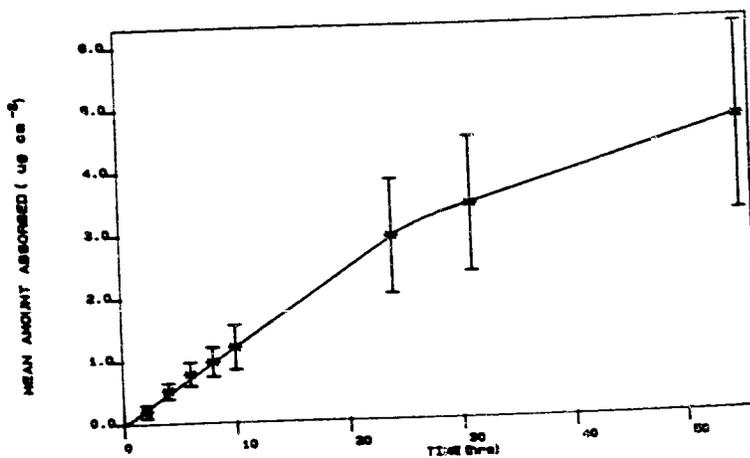
MEAN ABSORPTION PROFILE FOR
DISPERSE RED 60
FROM DYNASPERSE A THROUGH HUMAN EPIDERMIS
(n=5)



DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATION
THROUGH HUMAN AND PIG EPIDERMIS

FIGURE 7

MEAN ABSORPTION PROFILES FOR
DISPERSE YELLOW 3
FROM REAX 85A THROUGH PIG EPIDERMIS
(n=6)





Ecological and Toxicological Association of Dyes and Organic Pigments Manufacturers
U.S. DYE MANUFACTURERS OPERATING COMMITTEE OF ETAD

August 5, 1996

TO: USOC Toxicology Task Force

Skin Penetration Studies (ETAD Project T 2030)

For your information, attached is a correction to the Zeneca Report No. CTL/L/6841 which was included in the skin penetration reports circulated to you in the package mailed on June 19, 1996.

Tucker Helmes, Ph.D.
Executive Director

Attachment

File: T 2030

US/TOX/4

0036

ZENECA CENTRAL TOXICOLOGY LABORATORY
ALDERLEY PARK MACCLESFIELD
CHESHIRE UK

CATEGORY B REPORT (CONFIDENTIAL)
Not to be Copied Except by a
Reports Centre

Sponsor: ETAD
Sponsor Ref: T2030/PHASE 2
CTL Ref: Y08529/001 to 006
Y08538/001 to 006
CTL Study No: JV1445
Copy No:

REPORT NO: CTL/L/6841

FIRST AMENDMENT TO

DISPERSE RED 60 AND DISPERSE YELLOW 3:
IN VITRO ABSORPTION FROM SYNTHETIC PERSPIRATION
AND FIVE FORMULATIONS THROUGH HUMAN AND PIG EPIDERMIS

by

R J Ward

THE DATA IN THIS REPORT HAVE NOT BEEN QUALITY ASSURED

Approved for Issue:

M D J Padgham
M D J Padgham
Contract Manager

Date of Issue:

17 JUL 1982

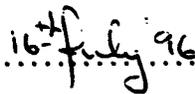
FIRST AMENDMENT TO CTL/L/6841

FIRST AMENDMENT TO

DISPERSE RED 60 AND DISPERSE YELLOW 3: IN VITRO ABSORPTION
FROM SYNTHETIC PERSPIRATION AND FIVE FORMULATIONS
THROUGH HUMAN AND PIG EPIDERMIS

In the above report, page 6, paragraph 1, line 4, the reference to Figure 3
should be to Figure 2.

Signature..........
R J Ward
(Study Director)

Date..........

detected until after 10 hours after application (Figure 2), after which time an absorption rate of $0.020\mu\text{g cm}^{-2}\text{ hr}^{-1}$ was achieved. From the $200\mu\text{l cm}^{-2}$ application of disperse yellow 3 (Table 1) absorption was measurable after 2 hours (Figure 2) at a rate of $0.090\mu\text{g cm}^{-2}\text{ hr}^{-1}$.

The mean absorption rate obtained for the disperse red 60 applied at $200\mu\text{l cm}^{-2}$ was the same as that obtained from an earlier study (Report No CTL/L/5926), when the dyes were applied at $1000\mu\text{g ml}^{-1}$ in water containing 0.5% TWEEN 80. The mean rate obtained for disperse yellow 3 was approximately twice the rate obtained in the earlier study.

4.2 Absorption from Formulations

No absorption ($<0.001\mu\text{g cm}^{-2}\text{ hr}^{-1} \approx 0.02\%$ of applied dose at 55hr) of disperse red 60 (Table 2) was detected from the Reax 85 formulation or of disperse yellow 3 (Table 3) from either the Reax 85 or Dynasperse A through human epidermis. The absorption rate for disperse red 60 from Dynasperse A was $0.003\mu\text{g cm}^{-2}\text{ hr}^{-1}$ (0.07% of applied dose at 55hr).

Mean absorption of both dyes from the Ultrazine NA and Ufoxane 2 formulations were the same ($0.006\mu\text{g cm}^{-2}\text{ hr}^{-1}$; 0.13% of applied dose at 55hr). The fastest absorption rates for disperse red 60 were from these Ultrazine NA and Ufoxane 2 formulations, but for disperse yellow 3, the fastest rate was from the Sodium Dispersol formulation ($0.012\mu\text{g cm}^{-2}\text{ hr}^{-1}$; 0.26% of applied dose at 55hr).

From the application of the Reax 85 formulation to pig epidermis the maximum absorption rate was measured between 1-24hr ($0.122\mu\text{g cm}^{-2}\text{ hr}^{-1}$). The average rate over 55hr was $0.087\mu\text{g cm}^{-2}\text{ hr}^{-1}$ (2% of applied dose at 55hr).

These results indicate that the absorption of these dyes, although very slow, was affected by the presence of the dispersing agents and that their effect on absorption rate was not necessarily the same for each of the two dyes tested.



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STUDY TITLE

¹⁴C-Mannitol, ³H-Water and Three Dyes: In Vitro Skin Penetration Following
a Single Application to the Excised Skin of Humans and Pigs

TEST SUBSTANCES

¹⁴C-Mannitol, ³H-Water,
¹⁴C-Disperse Blue 79, Reactive Blue 19 and Direct Blue 218

DATA REQUIREMENT

Not Applicable

AUTHORS

J. D. Sun, J. L. Beskitt and L. A. Zourelis

STUDY COMPLETION DATE

May 2, 1995

PERFORMING LABORATORY

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LABORATORY PROJECT ID

92N1108

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of the Dyestuffs Manufacturing Industry (ETAD)
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Washington, DC 20005

Page 1 of 44

UNION CARBIDE CORPORATION

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¹⁴C-Mannitol, ³H-Water and Three Dyes: In Vitro Skin Penetration Following a Single Application to the Excised Skin of Humans and Pigs

SUMMARY

The cutaneous penetration kinetics of ³H-water (550 μl) or ¹⁴C-mannitol (550 μl; 1 mg/ml) through excised pig ear and human abdominal skin samples were measured using in vitro techniques for a 6-hr period. In addition, the cutaneous penetration kinetics of ¹⁴C-Disperse Blue 79 (110 μl; 5 mg/ml), Reactive Blue 19 (550 μl; 1 mg/ml) and Direct Blue 218 (550 μl; 1 mg/ml) were evaluated by the same in vitro techniques using human abdominal skin samples. The CAS numbers were 2580-78-1 (Reactive Blue 19), 10401-50-0 (Direct Blue 218), 3618-72-2 (Dispersion Blue 79), and 69-65-8 (D-Mannitol). Test substances penetrating skin samples and collected in the effluent were measured by liquid scintillation spectrometry or high-performance liquid chromatography (HPLC).

For pig ear skin, the lag time before skin penetration of ¹⁴C-mannitol or ³H-water reached a steady-state rate was 2.77 and 1.75 hr, respectively. The steady-state rate of penetration of mannitol or water through pig ear skin was calculated to be 0.2 μg/cm²/hr and 6.12 mg/cm²/hr, respectively, while the Kp value was calculated to be 0.21 x 10⁻³ cm/hr and 6.12 x 10⁻³ cm/hr, respectively. These results were similar to those reported by Scott and Dick (1990). For human abdominal skin, the lag time before skin penetration of ¹⁴C-mannitol or ³H-water reached a steady-state rate was 3.49 and 2.17 hr, respectively. The steady-state rate of penetration of mannitol or water through human skin was calculated to be 0.006 μg/cm²/hr and 2.43 mg/cm²/hr, respectively, while the Kp value was calculated to be 0.006 x 10⁻³ cm/hr and 2.43 x 10⁻³ cm/hr, respectively.

These results show that significantly more ³H-water can penetrate both pig ear skin and human abdominal skin than ¹⁴C-mannitol. However, the total penetration of water and mannitol after 6 hr of exposure was 2.7-times and 55-times less for human skin than for pig ear skin. Similarly, both the steady-state penetration rates and permeability constants for water and mannitol were approximately 2.5-times and 30-times less, respectively, for human skin than for pig ear skin. These results would suggest that pig ear skin is not a good animal model for human cutaneous penetration of chemicals.

For the ¹⁴C-DB-79 applied to human abdominal skin, the lag time before skin penetration reached a steady-state rate was 3.38 hr. The steady-state rate of penetration of DB-79 through human skin was calculated to be 0.0057 μg/cm²/hr. The Kp value for the skin penetration of this dye, however, could not be calculated. Skin penetration of the Reactive Blue 19 and Direct Blue 218 applied to human abdominal skin could not be detected by HPLC above the minimum detection limit of 1 μg/ml.

Little or no Disperse Blue 79, Reactive Blue 19 or Direct Blue 218 was detected penetrating human skin samples. These results indicate that human dermal exposure to any of these 3 dye compounds would probably not result in a significant absorbed dose.

0 0 4 3

BACKGROUND INFORMATION

Cutaneous penetration of a potentially toxic chemical can be an important factor in the assessment of human health risk if skin contact commonly occurs during the manufacture or use of that compound. Cutaneous absorption of chemicals has been studied using various animal models. However, compared to other routes of exposure, little is known about the absorbed dose and the skin penetration rate of most chemicals following dermal exposure. Because of this, many risk assessments of dermal exposure to chemicals make the assumption that the entire exposure amount is absorbed into the body. This conservative approach would obviously over estimate the human health risk from such exposures. In addition, predictions of potential human health risk from results of dermal studies conducted in animals may not be an accurate reflection of the actual dose absorbed by humans.

In recent years, in vitro techniques have been developed to assess the cutaneous penetration kinetics of chemicals using a wide variety of animal and human skin samples. Studies using such techniques have allowed more accurate extrapolations of in vivo animal studies to human exposure scenarios and thus, provide more accurate assessments of potential human health risk following dermal exposure to chemicals.

OBJECTIVE

One purpose of these studies was to develop and validate an in vitro skin penetration measurement system that could be maintained as a resource in a U.S. laboratory for use by U.S. dye manufacturers to study the skin penetration of dye compounds. Therefore, the cutaneous penetration kinetics of ^3H -water or ^{14}C -mannitol through excised pig ear and human abdominal skin samples were determined. The results were then compared to similar findings from other laboratories in order to validate the methodologies used in the studies presented in this report. In addition, the cutaneous penetration kinetics of 3 dye compounds of interest to the members of the United States Operating Committee of ETAD were evaluated by the same in vitro techniques using human abdominal skin samples.

DOSE SELECTION

A volume of 550 μl of ^3H -Water or ^{14}C -Mannitol (1 mg/ml water) was applied to pig ear skin. The same was done for human skin, plus 550 μl of Reactive Blue 19 (1 mg/ml water), 550 μl of Direct Blue 218 (1 mg/ml water), or 110 μl of ^{14}C -Disperse Blue 79 (5 mg/ml acetone) was also tested (for the latter test substance, the acetone was evaporated as quickly as possible after application to the skin). For comparative purposes, these dose amounts and volumes were based on the doses used in similar studies conducted by another laboratory (Scott and Dick, 1990).

MATERIALS AND METHODS

The protocol and protocol amendments (BRRC Project ID 92N1108) detailing the design and conduct of this study are included in Appendix 1.

Unlabeled Test Substances

A sample of 100 g of Reactive Blue 19, Reference No. 1406SM-127B, CAS No. 2580-78-1, was received on November 2, 1993 from Hoechst Celanese, Coventry, RI, and assigned BRRC Sample No. 56-400. A 1 pint sample of Direct Blue 218, Lot No. 182, CAS No. 10401-50-0, was received on December 21, 1993 from Fabricolor, Paterson, NJ, and assigned BRRC Sample No. 56-451. The test substances were dark powders and were stored at room temperature.

A 5 gallon drum of Disperse Blue 79, Lot No. KA0008, CAS No. 3618-72-2, was received on January 10, 1990 from Sandoz Chemicals, Charlotte, NC, and assigned BRRC Sample No. 53-010. The test substance was a dark green powder and was stored at room temperature.

A 50 g sample of D-Mannitol, Lot No. TZ 00701TZ, CAS No. 69-65-8, was received on June 3, 1994 from Aldrich Chemical Company, Metuchen, NJ and assigned BRRC Sample No. 57-145. The test substance was a white powder and was stored at room temperature.

Labeled Test Substances

A sample of 5.0 mCi of ^3H -Water, Lot No. 2870-041, was received on June 2, 1992, from NEN Research Products, Boston, MA, and assigned BRRC Sample No. 55-150. The specific activity was 1.0 mCi/g. The test substance was a clear liquid and was stored refrigerated.

A sample of 100 μCi of ^{14}C -D-Mannitol, Lot No. 043H9216, was received on April 2, 1992, from SIGMA Chemical Company, St. Louis, MO, and assigned BRRC Sample No. 57-107. The specific activity was 51.6 mCi/mmol. The test substance was a clear liquid and was stored refrigerated.

A sample of 2.5 mCi of ^{14}C -C. I. Disperse Blue 79, Lot No. 2729-035, was received on June 5, 1990, from NEN Research Products, Boston, MA, and assigned BRRC Sample No. 53-191. The specific activity was 13.4 mCi/mM or 21 $\mu\text{Ci}/\text{mg}$. The radiochemical purity of this labeled test substance was determined at BRRC prior to its use to be approximately 89.1% pure. The test substance was a blue solution and was stored in the freezer.

Pig Skin Samples

Three female pig ears were obtained fresh from a local slaughterhouse and transported to the laboratory on wet ice.

Human Skin Samples

Fresh samples of human abdominal skin were obtained from IIAM (International Institute for the Advancement of Medicine), Exton PA. The samples were in physiological saline (0.9% NaCl) and shipped on wet ice. Upon arrival, the specimens were maintained in saline until prepared and placed in the chamber.

The initial experiment was conducted on June 10, 1994 and the final experiment on December 6, 1994.

Administration of Test Substances

Preparation of Skin

Skin discs were prepared by a modification of the method described by Kao *et al.* (1983) for full-thickness excised skin preparations. The hair of the pig ears was clipped and the ear separated (the outer portion was used). Skin pieces were gently scraped with a spatula or scalpel to remove fat and connective tissue. Three 1-inch discs from each pig ear and 10 1-inch discs from human skin specimens were placed in a Petri dish containing minimum essential medium (MEM; Eagle's medium with Earle's salts (Eagle, 1959), 25 mM HEPES buffer (Gibco) and penicillin/streptomycin as antimicrobial agents) (pig ear) or saline (human skin) prior to positioning in the chamber.

Application of Dose

A volume of 550 μ l of 14 C-mannitol (1 mg/ml) or 3 H-water was applied to 1 skin disc from each animal preparation and to 2 skin discs from each human skin preparation. Target radioactivity levels were 5-10 μ Ci per skin disc. The level of radioactivity in dosing solutions was determined by liquid scintillation spectrometry (LSS).

The Reactive Blue 19 and Direct Blue 218 dose solutions were prepared by weighing approximately 0.01 g of each dye into a 10 ml volumetric flask and diluting to volume with saline. A volume of 550 μ l of each 1 mg/ml solution was applied to 2 skin discs from each human skin preparation.

The 14 C-DB-79, in acetone, stock solution was at a concentration of 5 mg/ml. A volume of 110 μ l of the stock solution was applied to 2 skin discs from each human skin preparation and the acetone was immediately evaporated with a stream of nitrogen, to achieve a similar mass dose as the other 2 dye compounds tested. Target radioactivity levels were 5-10 μ Ci per skin disc. The level of radioactivity in dosing solutions was determined by LSS.

In all cases, the amount of test substance applied to each skin disc was an amount that covered the entire exposed skin surface (1.77 cm²) and to insure that an "infinite dose" was achieved. An "infinite dose" or an amount of test substance that would not be entirely absorbed through the skin during the 6-hr exposure period was used to allow the calculation of steady-state penetration rates and permeability constants (Kp) as described below. The opening in each chamber was sealed with a glass stopper to prevent evaporative losses during the experiments.

In Vitro Methodology

The apparatus and techniques used are a modification of the methods and skin chamber design previously described by Holland *et al.* (1984). A schematic representation of the current chamber design is shown in Figure 1. The dermal surface of the skin preparations was bathed with the MEM (pig ear) or saline (human skin) at a flow rate of approximately 2.5 ml/hr for at least 30 minutes prior to application of test substance. The test substances were applied to

the exposed epidermal surface (1.77 cm²) of each skin disc through the openings in the upper plate of the chamber. During the 0-6 hr sampling, media or saline effluent, at a flow rate of approximately 2.5 ml/hr, was voided directly into empty scintillation vials. The samples containing a radiolabeled test substance were then mixed with scintillation cocktail and analyzed by LSS. At termination of the ³H-water and ¹⁴C-mannitol experiments, skin pieces were removed from the chamber, placed in a Petri dish, and any unabsorbed dose was removed from the skin using water-wetted cotton swabs, which were placed in scintillation vials. Rinse samples were counted by LSS and skin samples were combusted in a biological oxidizer prior to LSS analysis for inclusion in the calculation of total recovery.

The effluent samples for the unlabeled test substances were collected in scintillation vials, weighed and then transferred to chromatographic vials for chemical analysis. No additional samples were collected or analyzed for the 3 dye compound experiments.

Sample Analysis

¹⁴C-Mannitol, ³H-Water, ¹⁴C-Disperse Blue 79

The amount of ¹⁴C-mannitol, ³H-water or ¹⁴C-Disperse Blue 79 that penetrated human skin samples and into effluent samples was directly measured by LSS. For this study, the average background radioactivity was approximately 50 DPM. The minimum detection limit of these radiolabeled compounds was set to twice this background level or 100 DPM.

Reactive Blue 19

To determine the amount of Reactive Blue 19 (BBR) that penetrated human skin samples into the effluent, procedures for the quantitation of BBR in saline were modified from methods obtained from the sponsor and validated at BRRC. Standard solutions of BBR in saline, ranging from 0.1-10 µg/ml, were prepared and analyzed using a high-performance liquid chromatograph (HPLC) equipped with a C-18 reverse-phase column and coupled to an ultraviolet detector. Table 1 describes the operating conditions used for these analyses.

An aliquot of each effluent sample was transferred to an HPLC vial for analysis. One hundred µl of each sample and standard were injected onto the HPLC system. The HPLC analysis of standard solutions of BBR in saline yielded a single BBR peak. The lower limit of detection was estimated to be 1 µg/ml.

Direct Blue 218

To determine the amount of Direct Blue 218 (DB-218) that penetrated human skin samples into the effluent, procedures for the quantitation of DB-218 in saline were modified from methods obtained from the sponsor and validated at BRRC. Standard solutions of DB-218 in saline, ranging from 0.1-10 µg/ml were prepared and analyzed using a HPLC equipped with a C-18 reverse-phase column and coupled to an ultraviolet detector. Table 2 describes the operating conditions used for these analyses.

An aliquot of each effluent sample was transferred to an HPLC vial for analysis. One hundred μ l of each sample and standard were injected onto the HPLC system. The HPLC analysis of standard solutions of DB-218 in saline yielded a single DB-218 peak. The lower limit of detection was estimated to be 1 μ g/ml.

Data Processing

Mass Balance Determinations

The total recovery of the applied radioactive dose was determined at the termination of the ^3H -water and ^{14}C -mannitol experiments. This included the amount of radiolabel measured in effluent samples, the amount of unabsorbed dose including the amount that could be rinsed from the skin surface, and the amount of residual chemical left in the skin which was inaccessible to removal by the water rinse. Mass balance determinations were not performed for the 3 dye compounds tested in this study.

Skin Penetration Characteristics

The amount of radiolabelled water and mannitol that penetrated skin (cumulative percent absorbed) was determined from the sum of effluent counts divided by the mean dosing solution counts. The steady-state penetration rate was computed by plotting interval ^{14}C or ^3H values (normalized to mass per unit surface area) vs. time and then taking the slope from the linear portion of the curve. These slope values were used to calculate permeability constant (k_p) values as described by Bronaugh *et al.* (1982). Figure 2 illustrates the concepts used for these calculations. Similar calculations to characterize the cutaneous penetration of the three dye compounds tested in this study could not be performed due to the small amounts of penetration.

RETENTION OF RECORDS

All raw data, documentation, the protocol, amendments, and a copy of the final report generated as a result of this study will be retained in the BRRC/UCC Archives for at least 10 years.

RESULTS AND DISCUSSION

Material Balance Determinations

Pig Ear Skin

Application of ^{14}C -mannitol to female pig ear skin resulted in 0.22% of the applied ^{14}C dose being recovered in media effluent samples (Table 3) after 6 hr. The largest recovery fraction for this compound was the unabsorbed dose residue (60.0%) removed from the skin, which included a water rinse of the skin surface. More of the dose was recovered from the skin disc after it was combusted (3.5%). A rinse of the apparatus recovered an additional 0.07% of

the applied dose. The average recovery was $63.7 \pm 4.5\%$ for the 3 pig ear skin samples tested.

For ^3H -water, about 8.4% of the radiolabel was recovered in media effluent samples by the end of the 6-hr measurement period (Table 3). Most of the dose was recovered as unabsorbed residue (56.8%) removed from the skin at the end of the experiment, which includes the water rinse. An additional 0.08% was recovered after combusting the skin samples. A rinse of the apparatus recovered an additional 1.2% of the applied dose. An average of 66.4% of the applied radioactivity was recovered for the 2 pig ear skin samples evaluated.

Human Abdominal Skin

Application of ^{14}C -mannitol to female human abdominal skin resulted in 0.003% of the applied ^{14}C dose being recovered in effluent samples (Table 4) after 6 hr. The largest recovery fraction for this compound was the unabsorbed dose residue (64.9) removed from the skin, which included a water rinse of the skin surface. More of the dose was recovered from the skin disc after it was combusted (0.8%). A rinse of the apparatus recovered an additional 0.06% of the applied dose. The average recovery was $65.8 \pm 8.6\%$ for the 3 human abdominal skin samples tested.

For ^3H -water, about 3.1% of the radiolabel was recovered in effluent samples by the end of the 6-hr measurement period (Table 4). Most of the dose was recovered as unabsorbed residue (60.3%) removed from the skin at the end of the experiment, which includes the water rinse. An additional 0.9% was recovered after combusting the skin samples. A rinse of the apparatus recovered an additional 0.5% of the applied dose. An average of $64.8 \pm 9.1\%$ of the applied radioactivity was recovered for the 3 human abdominal skin samples evaluated.

Mass balance determinations for the 3 dye compounds tested on human skin samples were not performed.

For both the pig ear skin and human skin experiments, the mass balance was consistently low. The reason for these low recoveries is not known, but since an "infinite dose" was maintained for all experiments, these low recoveries should not affect the penetration characteristics of these test substances or the interpretation of the data.

Skin Penetration Characteristics

Pig Ear Skin

When ^{14}C -mannitol was applied to pig ear skin, the lag time before skin penetration reached a steady-state rate was calculated to be 2.77 hr. The steady-state rate of penetration of mannitol through pig ear skin was calculated to be $0.2 \mu\text{g}/\text{cm}^2/\text{hr}$, while the K_p value was calculated to be $0.21 \times 10^{-3} \text{ cm}/\text{hr}$ (Table 5).

For the ^3H -water applied to pig ear skin, the lag time before skin penetration reached a steady-state rate was calculated to be 1.75 hr. The steady-state

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rate of penetration of mannitol through pig ear skin was calculated to be 6.12 mg/cm²/hr, while the Kp value was calculated to be 6.12 x 10⁻³ cm/hr (Table 5).

Scott and Dick (1990) conducted a similar study that measured the penetration of mannitol and water through full-thickness, pig ear skin. They reported Kp values for mannitol and water penetration to be 0.679 x 10⁻³ cm/hr and 2.25 x 10⁻³ cm/hr, respectively. These values are similar to those measured in this study and indicate that the methodology used in this study were comparable to those used by Scott and Dick.

Human Abdominal Skin

When ¹⁴C-mannitol was applied to human abdominal skin, the lag time before skin penetration reached a steady-state rate was calculated to be 3.49 hr. The steady-state rate of penetration of mannitol through human skin was calculated to be 0.006 µg/cm²/hr, while the Kp value was calculated to be 0.006 x 10⁻³ cm/hr (Table 6).

For the ³H-water applied to human abdominal skin, the lag time before skin penetration reached a steady-state rate was calculated to be 2.17 hr. The steady-state rate of penetration of water through human skin was calculated to be 2.43 mg/cm²/hr, while the Kp value was calculated to be 2.43 x 10⁻³ cm/hr (Table 6).

For the ¹⁴C-DB-79 applied to human abdominal skin, only 0.004% of the applied dose was found in effluent samples. The lag time before skin penetration reached a steady-state rate was calculated to be 3.38 hr. The steady-state rate of penetration of DB-79 through human skin was calculated to be 0.0057 µg/cm²/hr (Table 7). The Kp value for the skin penetration of this dye, however, could not be calculated because the acetone solvent was evaporated shortly after application. Therefore, an initial concentration of DB-79 was not available for the Kp equation (see Fig. 2).

For the BBR applied to human abdominal skin, none of the effluent samples contained peaks with the retention time of BBR (approximately 25.5 minutes) as measured by HPLC at a minimum detection limit of approximately 1 µg/ml. Therefore, the skin penetration kinetics of BBR could not be calculated.

For the DB-218 applied to human abdominal skin, HPLC analysis of the effluent samples yielded chromatography peaks having approximately the same retention time as DB-218 (approximately 3.7 minutes) that exceeded the highest concentration of the DB-218 standards. However, all of these effluent samples appeared colorless. By visual examination of the DB-218 standard solutions, all the standards were visibly blue in color, with the exception of the 0.5 µg/ml standard, and differed only in intensity. The 1 µg/ml standard had a slightly blue cast, the 5 µg/ml standard was a light blue and the 10 µg/ml standard was a darker blue. Again, the 0.5 µg/ml standard appeared colorless. From this, it was determined that the peaks seen by HPLC were interfering peaks arising from the skin samples and did not represent DB-218. Therefore, all effluent samples were assumed to contain less than 1 µg/ml of DB-218, the

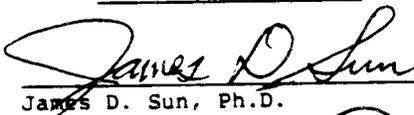
minimum detection limit, and because of this, the skin penetration kinetics of DB-218 could not be calculated.

CONCLUSIONS

Significantly more ^3H -water can penetrate both pig ear skin and human abdominal skin than ^{14}C -mannitol. Similar results were found in previous studies by Scott and Dick (1990). The difference between the permeability (k_p) of these two compounds was approximately 400-fold for human skin and 30-fold for pig ear skin. When these results are compared between species, it was found that the total penetration of water and mannitol after 6 hr of exposure was 2.7-times and 55-times less, respectively, for human skin than for pig ear skin. Similarly, both the steady-state penetration rates and permeability constants for water and mannitol were approximately 2.5-times and 30-times less, respectively, for human skin than for pig ear skin. These results would suggest that pig ear skin is not a good animal model for human cutaneous penetration of chemicals.

Disperse Blue 79, Reactive Blue 19 and Direct Blue 218 were also tested for their ability to penetrate human abdominal skin. For Disperse Blue 79, skin penetration was low and similar to that of mannitol, but was significantly less than that of water. No skin penetration of Reactive Blue 19 or Direct Blue 218 was detected above the minimum detection limit of 1 $\mu\text{g}/\text{ml}$. Therefore, under the conditions used in this study, these results indicate that human dermal exposure to any of these 3 dye compounds would probably not result in a significant absorbed dose.

REVIEW AND APPROVAL

Study Director:	 _____ James D. Sun, Ph.D.	5-2-95 _____ Date
Director:	 _____ John P. Van Miller, Ph.D., DABT	5/3/95 _____ Date

KEY PERSONNEL

Technical Coordinator: J. L. Beskitt

Scientist: L. A. Zourelis

Additional personnel are listed in the raw data.

REFERENCES

Bronaugh, R. L., Stewart, R. F., Congdon, E. R., and Giles, A. L. (1982). Methods for in vitro percutaneous absorption studies. I. Comparison with in vivo results. Toxicol. Appl. Pharm. 62, 474-480.

Eagle, H. (1959). Amino acid metabolism in mammalian cell culture. *Science* 130, 432.

Holland, J. M., Kao, J. Y., and Whitaker, M. J. (1984). A multi-sample apparatus for kinetic evaluation of skin penetration in vitro: The influence of variability and metabolic status of the skin. *Toxicol. Appl. Pharm.* 72, 272-280.

Kao, J., Hall, J., and Holland, J. M. (1983). Quantitation of cutaneous toxicity: An in vitro approach using skin organ culture. *Toxicol. Appl. Pharm.* 68, 206-217.

Scott, R. C. and Dick, I. P. (1990). Pig Skin: A preliminary investigation of permeability properties. ICI Central Toxicology Laboratory Report No. CTL/L/3284.

TABLE 1

¹⁴C-MANNITOL, ³H-WATER AND THREE DYES: IN VITRO SKIN PENETRATION FOLLOWING
A SINGLE APPLICATION TO THE EXCISED SKIN OF HUMANS AND PIGS

HPLC OPERATING PARAMETERS-EFFLUENT ANALYSIS
OF REACTIVE BLUE 19

Autosamplers:	Hewlett-Packard Series 1050 Autosampler or Waters WIST 710B
Pumps:	Hewlett-Packard 1050 Series Pumping System or Waters 501 Solvent Delivery Systems
Column:	Ficosphere C18, 3 μ m (3 cm), Perkin Elmer, Norwalk, CT
Column Temperature:	Ambient
Mobile Phase:	Solvent A: 99.05% Water, 0.14% Triethylamine Solvent B: 94.86% Acetonitrile, 5% Water, 0.14% Triethylamine
Flow Rate:	1.0 ml/minute
Detectors:	Hewlett-Packard Series 1050 Variable Wavelength or Waters 484 Tunable Absorbance
Wavelength:	254 nm
Injection Volume:	100 μ l
Integrators:	Hewlett-Packard 3395 Integrator or Waters 746 Data Module
Retention Time (RRR):	Approximately 25.5 minutes
Estimated Limit of Detection:	1 μ g/ml

TABLE 2

¹⁴C-MANNITOL, ³H-WATER AND THREE DYES: IN VITRO SKIN PENETRATION FOLLOWING
A SINGLE APPLICATION TO THE EXCISED SKIN OF HUMANS AND PIGS

HPLC OPERATING PARAMETERS-EFFLUENT ANALYSES
OF DIRECT BLUE 218

Autosampler:	Hewlett-Packard Series 1050 Autosampler
Pump:	Hewlett-Packard 1050 Series Pumping System
Column:	Mova-pak [®] C ₁₈ (3.9 mm x 30 cm), Waters, Milford, MA
Guard Column:	Waters μBondapak [®] C ₁₈ Guard-Pak [®] Pre-Column Inserts
Column Temperature:	Ambient
Mobile Phase:	20 mM Tetrabutylammonium Phosphate, 45% Methanol, 55%
Flow Rate:	1.0 ml/minute
Detector:	Hewlett-Packard Series 1050 Variable Wavelength
Wavelength:	254 nm
Injection Volume:	100 μl
Integrator:	Hewlett-Packard 3395 Integrator
Retention Time (DB-218):	Approximately 3.7 minutes
Estimated Limit of Detection:	1 μg/ml

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TABLE 3
¹⁴C-MANNITOL, ³H-WATER AND THREE DYES: IN VITRO SKIN PENETRATION FOLLOWING
 A SINGLE APPLICATION TO THE EXCISED SKIN OF HUMANS AND PIGS

MATERIAL BALANCE RECOVERIES FOR PIG EAR SKIN

Sample Analysed	Percent of Dose Recovered	
	¹⁴ C-Mannitol ^a	³ H ₂ O ^b
Effluents	0.22 ± 0.15	8.37
Unabsorbed Dose	59.98 ± 2.75	56.79
Apparatus Rinse	0.07 ± 0.05	1.20
Skin Combustion	<u>3.46 ± 2.18</u>	<u>0.08</u>
Total Recovery	63.73 ± 4.85	66.44

^a Mean ± 1 S.D. of 3 female pig ears.
^b Mean of 2 female pig ears.

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TABLE 4
¹⁴C-HANNITOL, ³H-WATER AND THREE DYES: IN VITRO SKIN PENETRATION FOLLOWING
 A SINGLE APPLICATION TO THE EXCISED SKIN OF HUMANS AND PIGS

MATERIAL BALANCE RECOVERIES FOR FEMALE HUMAN ABDOMINAL SKIN

Sample Analyzed	Percent of Dose Recovered	
	¹⁴ C-Hannitol ^a	³ H ₂ O ^b
Effluents	0.003 ± 0.006	3.08 ± 0.46
Unabsorbed Dose	64.93 ± 9.63	60.32 ± 9.24
Apparatus Rinse	0.06 ± 0.09	0.53 ± 0.19
Skin Combustion	<u>0.92 ± 1.45</u>	<u>0.97 ± 0.45</u>
Total Recovery	65.91 ± 8.57	64.80 ± 9.06

^a Mean ± 1 S.D. of 3 female human abdominal skins.

0 0 5 6

TABLE 5
¹⁴C-MANNITOL, ³H-WATER AND TRENE DYES: IN VITRO SKIN PENETRATION FOLLOWING
 A SINGLE APPLICATION TO THE EXCISED SKIN OF HUMANS AND PIGS

COMPARISON OF PERMEABILITY CONSTANTS FOR PIG EAR SKIN

Test Substance	Permeability Constant, k_p ^a ($\times 10^{-3}$ cm/hr)	Steady-State Penetration Rate ^b (mg/cm ² /hr)	Lag Time (hr)	Cumulative Dose Absorbed (%)
¹⁴ C-Mannitol ^c	0.21	0.0002	2.77	0.22
³ H ₂ O ^d	6.12	6.12	1.75	8.37

^a k_p = Steady State Penetration Rate (mg/cm²/hr); see footnote b.
 Initial dose concentration (mg/cm³)

^b Penetration rate at steady state, derived from the slope of the linear segment of a plot of the cumulative mg/cm² absorbed vs. time.

^c Mean of 3 pig ears.

^d Mean of 2 pig ears.

TABLE 6
¹⁴C-MANNITOL, ³H-WATER AND THREE DYES: *IN VITRO* SKIN PENETRATION FOLLOWING
 A SINGLE APPLICATION TO THE EXCISED SKIN OF HUMANS AND PIGS

COMPARISON OF PERMEABILITY CONSTANTS FOR FEMALE HUMAN ABDOMINAL SKIN

Test Substance	Permeability Constant, k_p ^a ($\times 10^{-3}$ cm/hr)	Steady-State Penetration Rate ^b (mg/cm ² /hr)	Lag Time (hr)	Cumulative Dose Absorbed (%)
¹⁴ C-Mannitol ^c	0.006 (0.007)	0.000006 (0.000008)	3.49 (0.52)	0.004 (0.004)
³ H ₂ O ^c	2.43 (0.23)	2.43 (0.23)	2.17 (0.63)	3.05 (0.16)

^a k_p = Steady State Penetration Rate (mg/cm²/hr); see footnote b.
 Initial dose concentration (mg/cm³)

^b Penetration rate at steady state, derived from the slope of the linear segment of a plot of the cumulative mg/cm² absorbed vs. time.

^c Mean (\pm 1 S.D.) of 3 female human abdominal skins.

TABLE 7
¹⁴C-MANNITOL, ³H-WATER AND THREE DYES: IN VITRO SKIN PENETRATION FOLLOWING
 A SINGLE APPLICATION TO THE EXCISED SKIN OF HUMANS AND PIGS

PENETRATION CHARACTERISTICS OF ¹⁴C-DB-79 FOR FEMALE HUMAN ABDOMINAL SKIN

Test Substance	Permeability Constant, k_p ($\times 10^{-3}$ cm/hr)	Steady-State Penetration Rate ^a ($\mu\text{g}/\text{cm}^2/\text{hr}$)	Lag Time (hr)	Cumulative Dose Absorbed (%)
¹⁴ C-DB-79 ^b	— c	0.0057	3.38	0.004

^a Penetration rate at steady state, derived from the slope of the linear segment of a plot of the cumulative mg/cm^2 absorbed vs. time.

^b Mean of 2 female human abdominal skins.

^c The k_p value could not be calculated because the acetone solvent was evaporated shortly after application. Therefore, an initial concentration of DB-79 was not available for the k_p equation (see Fig. 2).

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^{14}C -Mannitol, ^3H -Water and Three Dyes: *In Vitro* Skin Penetration Following a Single Application to the Excised Skin of Humans and Figs

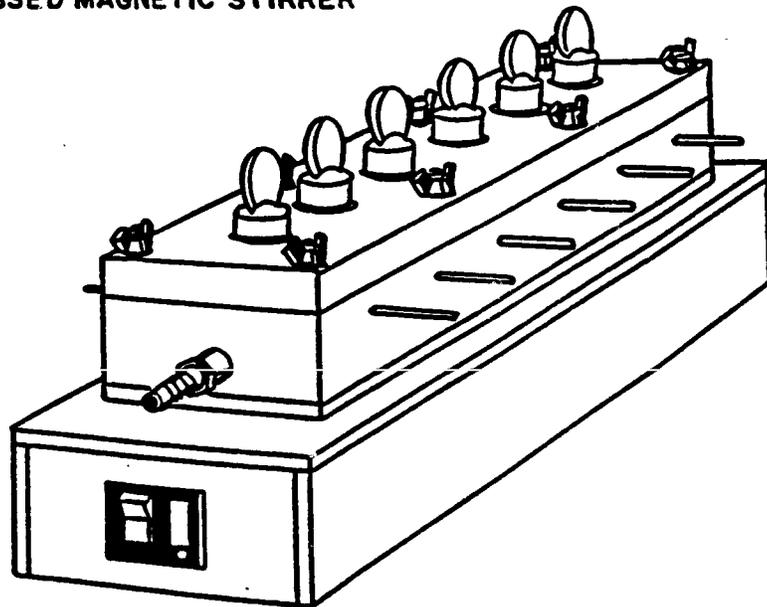
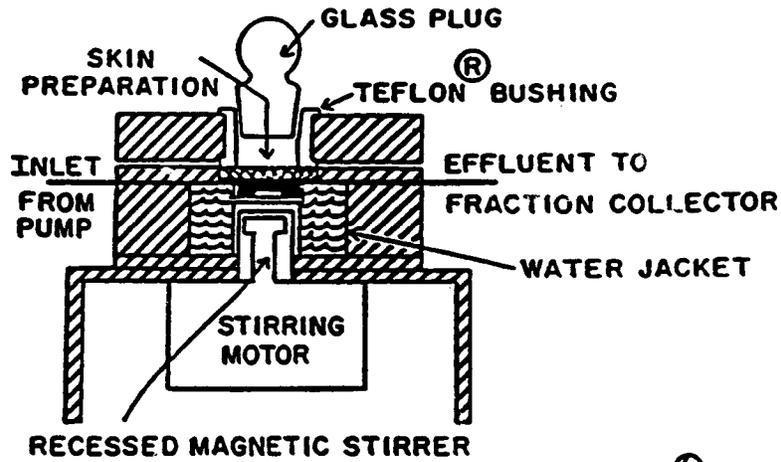
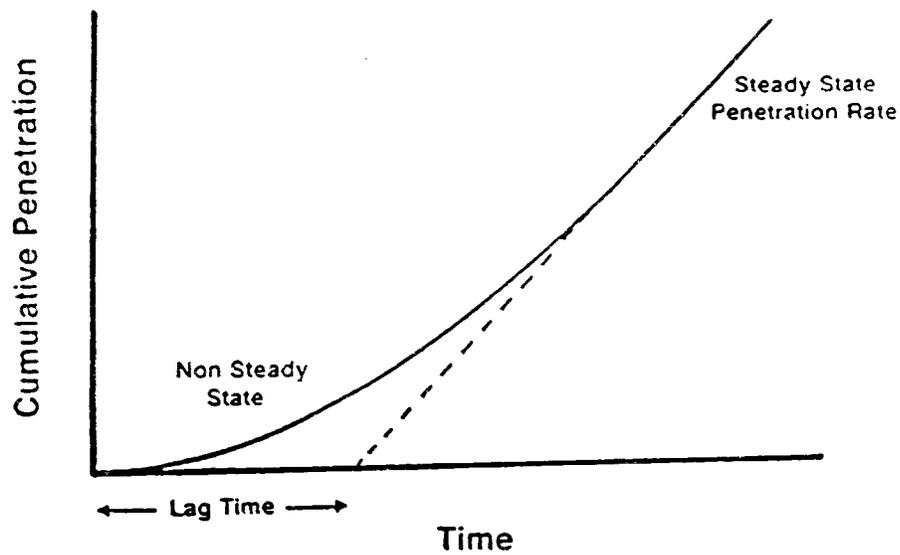


Figure 1 Schematic presentation of the skin penetration apparatus from Skin Permeation Systems (Berkeley, CA) used in this investigation.

¹⁴C-Mannitol, ³H-Water and Three Dyes: In Vitro Skin Penetration Following a Single Application to the Excised Skin of Humans and Pigs



$$\text{Permeability Constant } (k_p) = \frac{\text{Penetration Rate (mg/cm}^2\text{/hr)}}{\text{Initial Concentration (mg/cm}^3\text{)}}$$

Figure 2 A typical plot of mean cumulative skin penetration of a test or reference substance as function of time. Linear extrapolation to determine lag time and steady-state penetration rate, and calculation of a permeability constant (K_p) are also shown.

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14C-Mannitol, 3H-Water and Three Dyes: In Vitro Skin Penetration Following
a Single Application to the Excised Skin of Humans and Pigs

Protocol and Protocol Amendments

(22 Pages)

The Material Safety Data Sheet (Attachment 2)
that was included in the protocol has been removed due to
illegibility as a result of being reproduced for the report.



BUSHY RUN RESEARCH CENTER

6702 Mellon Road, Export, Pennsylvania 15632-8902

Telephone (412) 733-5200
Telecopier (412) 733-4804

PROTOCOL

TITLE: ^{14}C -Mannitol, ^3H -Water and Three Dyes: Species Comparisons of
In Vitro Skin Penetration Following a Single Application to the
Excised Skin of Humans and Pigs

BRRC PROJECT NUMBER: 92N1108

SPONSOR: Ecological and Toxicological
Association of the Dyestuffs
Manufacturing Industry (ETAD)
Suite 300
1330 Connecticut Avenue, NW
Washington, DC 20036

TESTING FACILITY: Bushy Run Research Center (BRRC)
Union Carbide Chemicals and
Plastics Company Inc.
6702 Mellon Road
Export, PA 15632-8902

Reviewed and Approved by:

Bushy Run Research Center:

Stephen W. Frantz 10/5/92
Stephen W. Frantz, Ph.D., DABT Date
Study Director

James D. Sun 10-5-92
James D. Sun, Ph.D. Date
Associate Director

John P. Van Miller 10-8-92
John P. Van Miller, Ph.D., DABT Date
Director

Sponsor's Representative:

C. Tucker Helms 10/16/92
C. Tucker Helms, Ph.D. Date
Executive Director, UBOC

Note: Bushy Run Research Center (BRRC) will conduct this study as a research project with the understanding that the study is not intended for submission to a regulatory agency. Therefore, it will not be audited by the BRRC Quality Assurance Unit prior to finalizing the study report.

Union Carbide Chemicals and Plastics Company Inc.
Excellence Through Quality

EQ

0063

OBJECTIVE

This study is designed to determine the time-course of cutaneous penetration and the recovery of radioactivity in effluent medium following application of ^{14}C -mannitol, ^3H -water and ^{14}C -labeled C. I. Disperse Blue 79:1 (DB79), to the excised skin of humans, in comparison with pig ear skin preparations. Also, this study will produce results which will compare/validate methodology for pig and human skin from other laboratories by generating permeability constants for ^3H -labeled water and ^{14}C -labeled mannitol. The initial phase will also evaluate ^{14}C -labeled DB79 on human skin, using ^3H -labeled water and ^{14}C -labeled mannitol as positive controls (cf. Attachment 1). Finally, the objective of this research project will be to compare these evaluations with 2 additional unlabeled dyes of interest to the members of the United States Operating Committee (USOC) of ETAD.

Design and Basis for the study

This study will consist of the evaluation of skin penetration for 2 standard chemicals and 3 dye compounds using human breast skin and pig ear skin.

The portions of this study conducted at ERRC will be conducted in the spirit of the following guidelines and standards, but no formal quality assurance will be conducted:

U.S. Environmental Protection Agency (EPA), Toxic Substances Control Act (TSCA) Good Laboratory Practice Standards, 40 CFR Part 792.

PERSONNEL

Biochemical Toxicology	J. L. Baskitt, A.S./MLT(ASCP) M. P. Fialkovich, B.S. S. W. Frantz, Ph.D., DABT J. D. Sun, Ph.D. M. J. Tallant, B.S.
Analytical Chemistry	E. M. Morris, M.S. B. E. Thomas, B.S. M. A. Vrbanic, B.A. L. A. Scurelias, A.S.
Clinical Veterinarian	E. H. Fowler, DVM, Ph.D., Diplomate ACVP

All personnel who participate in the conduct of the study will be documented in the raw data.

PROJECT DATES

<u>Starting Date of Acclimation</u>	To be added by amendment.
<u>Starting Date of Test Substance Administration</u>	To be added by amendment.
<u>Proposed Date for Submission of the Draft Final Report</u>	To be added by amendment, based on probe studies.

METHODS

Test Substance

Chemical Names	Mannitol, Water and C. I. Disperse Blue 79:1 (DB79)
CAS Registry Numbers	Mannitol - 69-65-8; water - 7732-18-5; and DB79 - 3618-72-2
Source	To be added by amendment.
Sponsor Identification Numbers	To be supplied by the Sponsor and added by amendment.
BRRC Numbers	To be added by amendment.
Description	To be added by amendment.
Purity	To be added by amendment.
Stability of Test Substance	To be supplied by the Sponsor and added by amendment.
Reference or Lot Numbers	To be added by amendment.
Radiochemical Test Substance	^{14}C -labeled mannitol and DB79, and $^3\text{H}_2\text{O}$ will be obtained from a commercial supplier. The specific activity and radiochemical purity will be targeted for approximately 15 mCi/mmol and $\geq 97\%$, respectively. Chromatographic documentation of the purity and identity of the ^{14}C -labeled test substance will be provided. Confirmation of radiochemical purity will be done at BRRC only at the Sponsor's request and at additional cost.
Molecular Formula and Weight	To be added by amendment.
Solubility in Water	To be added by amendment.
Storage Conditions	The test substance will be stored in the most appropriate manner to ensure stability.
Quantity	1.0 mCi of ^{14}C -radiolabeled test substance.
Reserve Sample	A reserve sample will not be retained unless requested by the Sponsor.

Safety

A Material Safety Data Sheet (MSDS) for DB79 supplied by the Sponsor will be reviewed by all personnel prior to the initiation of the study (Attachment 2). This review will be documented. Normal precautions for untested substances will be used. These procedures include the use of disposable Tyvek® or plastic coats or jumpsuits, hats, booties or shoe covers, and PVC gloves while in the animal rooms. Eye protection will include the use of safety glasses at all times. Disposable Tyvek® coats or smocks and PVC-coated gloves will be worn during use of the test substance. In addition, monogoggles will be used when handling the test substance.

Test Animals

Species and Strain

Pigs

Supplier

Local slaughterhouse.

**Number
and Sex**

At least 3 females.

Pig Skin Samples

Pig ear skin will be obtained from a local slaughterhouse. The specimens will be prepared from the outer ear and maintained in minimum essential medium (MEM/d-Valine)* upon removal until skin discs can be prepared and placed in the chamber.

Human Skin Samples

Freshly-obtained samples of human skin will be obtained from mammoplasty patients in the University of Pittsburgh Hospital system in collaboration with the Division of Plastic and Reconstructive Surgery, School of Medicine. This cooperative arrangement has been approved by the University's Institutional Review Board for Biomedical Research and both informed consent and a full demographic profile will be obtained from each patient prior to removal of the skin specimen. The specimens will be maintained in minimum essential medium (MEM/d-Valine)* upon removal until skin discs can be prepared and placed in the chamber.

**Preparation
of Skin**

Discs of skin will be prepared by a modification of the method described by Kao *et al.* (1983) for full-thickness excised skin preparation. Pig ears will be obtained from a local slaughterhouse. The outer ear region will be cleaned with water, carefully shaven and the skin removed by dissection. For processing of animal skin, a piece of clipped skin (approximately 6

*Eagle's medium with Earle's salts (Eagle, H. (1959) *Science*, 130, 432) and 25 mM HEPES buffer (Gibco); with penicillin/streptomycin as antimicrobial agents.

x 6 cm) will be removed and placed in a petri dish containing MEM to keep it moist; human skin will be received in MEM from the hospital and processed similarly to animal skin. The skin piece will be placed on a dissecting board and scraped with a spatula to remove fat and connective tissue. Two 1-inch discs from the animal skin piece and six 1-inch discs from human skin specimens will be removed and placed in a petri dish with several drops of MEM to keep moist before placing in the chamber.

Administration of Test Substance

Application of Dose

The radiolabeled test chemicals will be applied in an amount sufficient to produce a target radioactivity level of at least 5-10 $\mu\text{Ci/skin preparation}$; the actual concentration applied, in mg/cm^2 of skin surface, will be documented in the study records and final report. Dosing solutions will be analyzed only by quantification of radioactivity by liquid scintillation spectrometry.

Study Design

The following methodology will be used to conduct the experiments defined in the study design summary table contained in Attachment 1 to this protocol.

In Vitro Methodology

The technique used is a modification of the methods and skin chamber described by Holland *et al.* (1984) which has been verified by Frantz *et al.* (1991a) and successfully used on previous studies (Frantz *et al.*, 1991b; Tallant *et al.*, 1990). The skin specimens will be placed into the chamber and the dermal surface of the skin preparations will be perfused with MEM for organ culture as described by Kao *et al.* (1983) at an approximate flow rate of 2.5 mL/hr for at least 30 minutes prior to application of test substance. Test substances will be applied in a minimal volume of an appropriate vehicle to the exposed epidermal surface (2.0 cm^2) of each skin disc through the openings in the upper plate of the chamber. These experiments will be conducted for approximately 6 hr in order to demonstrate steady-state penetration rates. Media effluent will be voided directly into empty scintillation vials and liquid scintillation cocktail will be added to the effluent. These samples will then be counted for ^{14}C activity in a liquid scintillation spectrometer. The amount of test substance which penetrates will be presented as cumulative percent absorbed radioactivity determined from the sum of counts found in the effluent media divided by the mean dosing solution counts. The penetration rate will be computed by normalizing interval radioactivity values to an hourly rate and plotting these values at the endpoint of the measurement interval.

Balance Determination

Upon termination of the experiment, the skin pieces will be removed from the chamber and placed in a petri dish for further removal of the remaining dose; any unabsorbed dose will be removed (typically, using water-wetted, cotton swabs) from the skin and placed in a scintillation vial. The samples will be radioassayed by liquid scintillation spectrometry for inclusion in the calculation of total balance. The skin samples will be stored frozen until analyzed (typically, combusted directly in a biological oxidizer or pulverized at liquid N₂ temperatures in a freezermill).

Analytical Chemistry

Information on the composition and solubilities of the test substance will be provided by the Sponsor. Development of an analytical method for the specific characterization/identification of the chemical which penetrates into effluent media may be provided (at an additional cost to the Sponsor). Detailed description of the methods employed will be appended to the final report. Phase II development of analytical methods (cf. Attachment 1) will be completed before Phase III testing begins.

Data Processing

The amount of test substance which penetrates skin will be presented as cumulative percent absorbed radioactivity and will be determined from the sum of counts found in the effluent media divided by the counts determined for the amount of dosing solution applied to each skin preparation. The pseudo steady-state penetration rate will be determined from interval radioactivity values, which will be calculated as the cumulative mg/cm² absorbed, and an hourly rate will be taken from the linear segment of the curve after plotting these values versus time (data plotted at the endpoint of the measurement interval). Permeability constants (k_p) for each chemical will be calculated using the following formula as described by Bronaugh et al. (1982):

$$k_p \text{ (in cm/hr)} = \frac{\text{Steady State Absorption Rate (mg/cm}^2\text{/hr)}}{\text{Initial Dose Concentration (mg/cm}^3\text{)}}$$

Additional calculations of the projected amount of each chemical which would penetrate the preparations of skin at contact times beyond the 6-hr determination will be calculated using linear regression (corrected for surface area of absorption) to derive the amount absorbed (percent of dose) at the projected interval.

ALTERATION OF PROTOCOL

Alterations to this protocol may be made as the study progresses. No changes in the protocol will be made without the specific written request or consent

of the Sponsor. In the event that the Sponsor authorizes a protocol change verbally, such change will be honored. However, it then becomes the responsibility of the Sponsor to follow such verbal change with a written verification. BTRC reserves the right to revise the protocol or deviate therefrom solely at the discretion of the Study Director if prior approval of the Sponsor cannot be obtained and the integrity of the study is considered in jeopardy. In this event, the Sponsor shall be notified of the alteration as soon as possible, and written verification of the change will be the responsibility of the Study Director. All protocol modifications will be signed by the Study Director and a representative of the Sponsor.

RECORDS

All records pertaining to this study will be retained by BTRC for at least 10 years after completion of the study.

Prior to discarding any of the above data or materials, the Sponsor will be contacted and given the option of obtaining it or arranging for continued storage. All records and materials mentioned above will remain the sole property of the Sponsor and can be removed from BTRC at the Sponsor's discretion.

DISPOSAL OF RADIOACTIVE SAMPLES

Radioactive sample disposal will be conducted by methods which follow laboratory SOPs upon completion of the final report. The Sponsor will be notified prior to disposal of the samples.

REPORTS

Draft Report

A draft of the report will be submitted to the Sponsor approximately 3 months after completion of the final experiment. This report will be a comprehensive report which will include all information necessary to provide a complete and accurate description and evaluation of the test procedures and results. It will include: a summary; appropriate text discussions of the experimental design, materials and methods and results; summary tables of mean radioactive recoveries; and graphic representation of skin penetration. Three copies of the draft report will be provided to the Sponsor.

Final Report

The draft report will be reviewed by the Sponsor, and comments on the report will be provided to BTRC within 8 weeks from the date of submission of the draft version. BTRC will consider these comments in preparing the final report. Assuming the Sponsor's comments are received at the specified time and no major revisions are required, BTRC will submit a final report within 12 weeks of issuance of the draft report.

The final report will be written in manuscript form, with the expressed intention of submitting it for publication in a peer-reviewed journal. The Sponsor shall have a full opportunity to review the draft manuscript prior to its submission to journal approved of by the Sponsor.

REFERENCES

- Bronaugh, R. L., Stewart, R. F., Congdon, E. R., and Giles, A. L. (1982). Methods for in vitro percutaneous absorption studies. I. Comparison with in vivo results. *Toxicol. Appl. Phars.* 52, 474-480.
- Frantz, S. W., Dittenber, D. A., Eisenbrandt, D. L., and Watanabe, P. G. (1991a). Evaluation of a flow-through in vitro skin penetration chamber method using acetone-deposited organic solids. *J. Toxicology: Cutaneous & Ocular Toxicol.* 9(4), 277-299.
- Frantz, S. W., Tallant, M. J., and Ballantyne, B. (1991b). In vitro skin penetration of Polymer JR400: species comparisons following a single application to the excised skin of humans, Fischer 344 rats, B₆C₃F₁ mice, Hartley guinea pigs, and New Zealand White rabbits. *J. Toxicology: Cutaneous & Ocular Toxicol.* 10(3), 175-186.
- Holland, J. M., Kao, J. Y., and Whitaker, M. J. (1984). A multi-sample apparatus for kinetic evaluation of skin penetration in vitro: The influence of variability and metabolic status of the skin. *Toxicol. Appl. Pharmacol.* 72, 272-280.
- Kao, J., Hall, J., and Holland, J. M. (1983). Quantitation of cutaneous toxicity: An in vitro approach using skin organ culture. *Toxicol. Appl. Pharmacol.* 68, 206-217.
- Tallant, M. J., Frantz, S. W., and Ballantyne, B. (1990). Evaluation of the in vitro skin penetration potential of glutaraldehyde using rat, mouse, rabbit, guinea pig, and human skin. *The Toxicologist* 10, 256.

Study Design for In Vitro Skin Penetration Comparison of Excised Pig and Human Skin Samples

<u>Experiment</u>	<u>Numbers of Human & Pig Skin Samples</u>	<u>Description</u>
1. Definitive Pig Skin Investigation	3 female pigs, 2 preparations/pig	Definitive study with ¹⁴ C-Mannitol and ³ H-Water.
2. First Human Skin Sample	6 skin preparations per human sample	Evaluation of ¹⁴ C-DB79 plus ¹⁴ C-Mannitol and ³ H-Water.
3. Second Human Skin Sample	6 skin preparations per human sample	Evaluation of ¹⁴ C-DB79 plus ¹⁴ C-Mannitol and ³ H-Water.
4. Third Human Skin Sample	6 skin preparations per human sample	Evaluation of ¹⁴ C-DB79 plus ¹⁴ C-Mannitol and ³ H-Water.

Phase II

MFPC Validation Work - Methods for 2 dye formulations to be conducted in Phase III.

Phase III - PROPOSED

<u>Experiment</u>	<u>Numbers of Human Skin Samples</u>	<u>Description</u>
5. First Human Skin Sample	6 skin preparations per human sample	Evaluation of 2 dye * formulations & ¹⁴ C-Mannitol std.
6. Second Human Skin Sample	6 skin preparations per human sample	Evaluation of 2 dye * formulations & ¹⁴ C-Mannitol std.
7. Third Human Skin Sample	6 preparations per skin human sample	Evaluation of 2 dye * formulations & ¹⁴ C-Mannitol std.

* Specific dye candidates to be selected by the Sponsor and assumes available MFPC method for analysis of effluent dye concentrations.

Note: This design may be modified following consultation with the Sponsor regarding the results of the MFPC validation work. Such modifications will be formally amended to the study protocol.

NRRC-PROTOCOLS-FOR
September 11, 1992

The Material Safety Data Sheet (Attachment 2; Pages 10-13)
that was included in the protocol has been removed due to
illegibility as a result of being reproduced for the report.



BUSHY RUN RESEARCH CENTER

6702 Mellon Road, Export, Pennsylvania 15632-8902

Telephone (412) 733-5200
Telecopier (412) 733-4804

PROTOCOL AMENDMENT 1

TITLE: ^{14}C -Mannitol, ^3H -Water and Three Dyes: Species Comparisons of
In Vitro Skin Penetration Following a Single Application to the
Excised Skin of Humans and Pigs

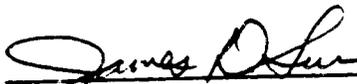
BRRC PROJECT ID: 92N1108

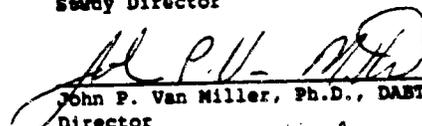
SPONSOR: Ecological and Toxicological
Association of the Dyestuffs
Manufacturing Industry (ETAD)
Suite 300
1330 Connecticut Avenue, NW
Washington, DC 20036

TESTING FACILITY: Bushy Run Research Center (BRRC)
Union Carbide Chemicals
and Plastics Company Inc.
6702 Mellon Road
Export, PA 15632-8902

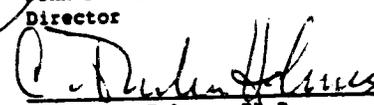
Reviewed and Approved by:

Bushy Run Research Center:

 9-17-93
James D. Sun, Ph.D. Date
Study Director

 9-21-93
John P. Van Miller, Ph.D., DABT Date
Director

Sponsor's Representative:

 9/30/93
C. Tucker Holmes, Ph.D. Date
Executive Director, USOC

Union Carbide Chemicals and Plastics Company Inc.
Excellence Through Quality



The protocol is amended as follows:

Item 1

Location of Protocol Change	Page 1, Reviewed and Approved by
Description of Protocol Change	James D. Sun replaces Stephen W. Frantz as the Study Director.
Rationale	Stephen W. Frantz is no longer employed at BRRC.

Item 2

Location of Protocol Change	Page 4, Human Skin Samples
Description of Protocol Change	Samples of human female abdominal skin will be obtained from post mortem donors through the International Institute for the Advancement of Medicine (IIAM, Exton, PA). In obtaining the IIAM tissues, a patient history with cause of death will be supplied. It will also be specified that the tissues must be fresh with regard to metabolic capacity. The specimens will be maintained in minimum essential medium (MEM/d-Valine)* upon receipt until skin discs can be prepared and placed in the chamber.
Rationale	A more consistent and reliable supply of human skin can be obtained from this source than is currently available from plastic surgery sources.

*Eagle's medium with Earle's salts (Eagle, H. (1959) Science, 130, 432) and 25 mM HEPES buffer (Gibco); with penicillin/streptomycin (100 ml of 5,000 units per 0.5 liter) added as antimicrobial agents.

BRRC/PROTOCOL/AMEND/1

0 0 7 4



BUSHY RUN RESEARCH CENTER

6702 Mellon Road, Export, Pennsylvania 15632-6902

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Telecopier (412) 733-4804

PROTOCOL AMENDMENT 2

TITLE: ^{14}C -Mannitol, ^3H -Water and Three Dyes: In Vitro Skin Penetration
Following a Single Application to the Excised Skin of Humans
and Pigs

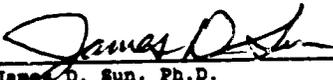
BRRC PROJECT ID: 92N1108

SPONSOR: Ecological and Toxicological
Association of the Dyestuffs
Manufacturing Industry (ETAD)
Suite 300
1330 Connecticut Avenue, NW
Washington, DC 20036

TESTING FACILITY: Bushy Run Research Center (BRRC)
Union Carbide Corporation
6702 Mellon Road
Export, PA 15632-6902

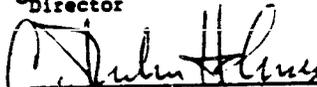
Reviewed and Approved by:

Bushy Run Research Center:


James D. Sun, Ph.D. 7-14-94
Date
Staff Director


John P. Van Miller, Ph.D., DABT 7/14/94
Date
Director

Sponsor's Representative:


C. Tucker Helmes, Ph.D. 7/27/94
Date
Executive Director, USOC

UNION CARBIDE CORPORATION

The protocol is amended as follows:

Item 1

Location of Protocol Change	Page 1, Title
Description of Protocol Change	¹⁴ C-Mannitol, ³ H-Water and Three Dyes: <u>In Vitro</u> Skin Penetration Following a Single Application to the Excised Skin of Humans and Pigs
Rationale	The title was changed to reflect changes to the study design which were requested by the Sponsor.

Item 2

Location of Protocol Change	Page 1, Testing Facility
Description of Protocol Change	The address for the testing facility has been changed to the following: Bushy Run Research Center (BRRC) Union Carbide Corporation 6702 Mellon Road Export, PA 15632-8902
Rationale	The corporate name was changed from Union Carbide Chemicals and Plastics Company Inc. to Union Carbide Corporation.

Item 3

Location of Protocol Change	Page 2, Objective
Description of Protocol Change	This study is designed to determine the <u>in vitro</u> skin penetration of ¹⁴ C-mannitol and ³ H-water and 3 dye compounds in excised pig ear and human skin preparations. Initial studies will involve the application of ¹⁴ C-mannitol and ³ H-water to excised pig ear skin preparations utilizing a flow-through <u>in vitro</u> skin penetration technique. Following this determination, an <u>in vitro</u> skin penetration study will be conducted using the same flow-through technique, for three dye compounds. The time course of cutaneous penetration in effluent medium after single applications of ¹⁴ C-C. I. Disperse Blue 79:1 (DB-79), Reactive Blue 19, and Disperse Blue 218 will be determined. Additionally, the percent of any radioactive doses applied will also be determined.

with human skin preparations

Direct

TW
7/27/94

TW
7/27/94

Rationale The objective was changed to reflect changes to the study design which were requested by the Sponsor.

Item 4

Location of Protocol Change Page 3, Test Substances, Chemical Names

Description of Protocol Change Reactive Blue 19 and Direct Blue 218

Rationale This information was not available at the time that the protocol was issued.

Item 5

Location of Protocol Change Page 3, Test Substances, CAS Registry Numbers

Description of Protocol Change Reactive Blue 19 - CAS Number 2580-78-1
Direct Blue 218 - CAS Number 10401-50-0

Rationale This information was not available at the time that the protocol was issued.

Item 6

Location of Protocol Change Page 3, Test Substances, Source

Description of Protocol Change Reactive Blue 19 - Hoechst Celanese, Coventry, RI
Direct Blue 218 - Fabricolor, Paterson, NJ
DB-79 - Sandoz Chemicals, Charlotte, NC
Radiolabeled DB-79 - NEN Research Products, Boston, MA
¹⁴C-Mannitol - Sigma Chemical Company, St. Louis, MO
³H-Water - NEN Research Products, Boston, MA

Rationale This information was not available at the time that the protocol was issued.

Item 7

Location of Protocol Change Page 3, Test Substances, Sponsor Identification Numbers

Description of Protocol Change Reactive Blue 19 - Reference Number 1406EM-127b
Direct Blue 218 - Lot Number 182
DB-79 - Lot Number KA0008
Radiolabeled DB-79 - Lot Number 2729-035
¹⁴C-Mannitol - Lot Number 043R9216
³H-Water - Lot Number 2870-041

Rationale This information was not available at the time that the protocol was issued.

Item 8

Location of Protocol Change

Page 3, Test Substances, BRRRC Numbers

Description of Protocol Change

Reactive Blue 19 - 56-400
Direct Blue 218 - 56-451
DB-79 - 53-010
Radiolabeled DB-79 - 53-191
¹⁴C-Mannitol - 57-107
³H-Water - 55-150

Rationale

This information was not available at the time that the protocol was issued.

Item 9

Location of Protocol Change

Page 3, Test Substances, Description

Description of Protocol Change

Reactive Blue 19 - black powder
Direct Blue 218 - black powder
DB-79 - dark green powder
Radiolabeled DB-79 - blue solution
¹⁴C-Mannitol - clear liquid
³H-Water - clear liquid

Rationale

This information was not available at the time that the protocol was issued.

Item 10

Location of Protocol Change

Page 3, Test Substances, Purity

Description of Protocol Change

Reactive Blue 19 - 76.7%
Direct Blue 218 - 90 to 90% dye with the bulk of the remainder being inorganic salts
DB-79 - 98.6%
Radiolabeled DB-79 - to be determined at BRRRC; results will be included in the raw data and final report.
¹⁴C-Mannitol - 98.0%
³H-Water - 99%

Rationale

This information was not available at the time that the protocol was issued. The purity of the Direct Blue 218 was to be supplied by the Sponsor, but this information was unavailable.

Item 11

Location of Protocol Change Page 3, Test Substances, Reference or Lot Numbers

Description of Protocol Change Not applicable

Rationale This information is included in the Sponsor Identification Numbers section; therefore, it is not applicable.

Item 12

Location of Protocol Change Page 3, Test Substances, Solubility in Water

Description of Protocol Change Reactive Blue 19, not available
Direct Blue 218, 30 g/L
DB-79, insoluble

Rationale This information was not available at the time that the protocol was issued. Although no specific numbers were provided, it was determined that the solubility of Reactive Blue 19 in water is sufficient for the proposed study. DB-79 was determined to be insoluble in water at BRRC. The concentration ranges will be stated in the raw data and included in the final report.

Item 13

Location of Protocol Change Page 4, Human Skin Samples

Description of Protocol Change The samples of human female skin will be maintained in 0.9% saline upon receipt until skin discs can be prepared and placed in the chamber.

Rationale Due to interfering peaks caused by the minimum essential medium (MEM) observed in the analytical method for Direct Blue 218, the effluent medium was changed from MEM to 0.9% saline.

Item 14

Location of Protocol Change Page 5, Preparation of Skin

Description of Protocol Change The 1-inch discs of human female skin will be placed in a petri dish with several drops of 0.9% saline to keep moist before placing in the chamber.

Rationale The effluent medium was changed from MEM to 0.9% saline for human skin.

Item 15

Location of Protocol Change Page 5, Study Design, Attachment 1

Description of Protocol Change See Attachment 1.

Rationale The study design was changed at the request of the Sponsor. The original design called for 6 human skin donors; the revised design can be done using 3 human skin donors.

Item 16

Location of Protocol Change Page 5, Administration of Test Substance, In Vitro Methodology

Description of Protocol Change The technique used is a modification of the methods described by Holland et al. (1984). The flow-through system consists of 6 individual wells in a chamber constructed primarily of transparent acrylic. Inlet and outlet ports allow for a continuous flow of effluent medium, which is continuously mixed by a mechanically-stirred, Teflon[®]-coated magnetic stir bar. The skin samples are held in place using an acrylic upper-plate, with a specially-designed Teflon[®]/O-ring housing for each of the wells. The skin specimens will be placed into the chamber and the fasteners simultaneously secured. For the pig ear experiments, MEM will be used as effluent medium, and saline will be used for human skin experiments. The dermal surface of the skin preparations will be bathed with effluent at an approximate flow rate of 2.5 ml/hr for at least 30 minutes prior to application of test substance. Temperature control will be provided by means of a manifold which runs the length of the chamber underside and is bathed continuously from a circulating water bath maintained at approximately 37°C. Test substances will be applied to the exposed epidermal surface (1.8 cm²) of each skin disc through the opening in the upper plate of the chamber. Media effluents will be voided directly into empty scintillation vials using a fraction collector, and an aliquot of the effluent will be analyzed.

Rationale This information was not available at the time that the protocol was issued.

Item 17

Location of
Protocol Change

Page 6, Data Analysis (Previously Analytical
Chemistry)

Description of
Protocol Change

To determine the amount of ^{14}C -mannitol and ^3H -water that penetrated each skin preparation, an aliquot of each effluent will be dissolved into liquid scintillation cocktail. These samples will then be counted for ^{14}C -activity in a liquid scintillation spectrometer (LSS).

To determine the amount of DB-79, Reactive Blue 19, and Direct Blue 218 that penetrated each skin preparation, an aliquot of each effluent will be analyzed by high performance liquid chromatography (HPLC). The analytical conditions will be optimized for adequate detection, resolution, and identification of each analyte. The analytical techniques for determining the concentration of each in effluent samples will be described in the raw data, and included in the final report. Any unexpected interferences in the matrix requiring additional methods development and/or reanalysis will be performed at additional cost to the Sponsor.

Rationale

Originally, all radiolabeled substances were to be analyzed by LSS. However, due to the known impurity of DB-79, effluents from the DB-79 will be analyzed using a method employing HPLC.

Item 18

Location of
Protocol Change

Page 5, Data Processing, ^{14}C -Mannitol and ^3H -Water

Description of
Protocol Change

The amounts of ^{14}C -mannitol and ^3H -water which penetrate skin will be presented as cumulative percent absorbed radioactivity and will be determined from the sum of counts found in the effluent media divided by the counts determined for the amount of dosing solution applied to each skin preparation. The steady-state penetration rate will be determined from interval radioactivity values, calculated as the cumulative $\mu\text{g}/\text{cm}^2$ absorbed, and an hourly rate will be taken from the linear segment of the curve after plotting these values versus time.

The amounts of DB-79, Reactive Blue 19, and Direct Blue 218 which penetrate the skin will be presented as amount of test substance per dose area ($\mu\text{g}/\text{cm}^2$) per interval, and cumulative $\mu\text{g}/\text{cm}^2$. The steady-state

penetration rate will be calculated as the cumulative mg/cm² absorbed/hr, and an hourly rate will be taken from the linear segment of the curve after plotting these values versus time.

Permeability constants (k_p) for each test substance will be calculated using the following formula (Bronaugh et al., 1982):

$$k_p \text{ (in cm/hr)} = \frac{\text{Steady State Absorption Rate (mg/cm}^2\text{/hr)}}{\text{Initial Dose Concentration (mg/cm}^2\text{)}}$$

Additional calculations of the projected amount that would penetrate the skin preparation at contact times beyond the 6-hour determination will be calculated using linear regression and corrected for surface area of absorption to derive the amount absorbed at the projected time point.

Rationale

This information was not available at the time that the protocol was issued.

REFERENCES

- Bronaugh, R. L., Stewart, R. F., Congdon, E. R., and Giles, A. L. (1982). Methods for in vitro percutaneous absorption studies. I. Comparison with in vivo results. *Toxicol. Appl. Pharm.* **62**, 474-480.
- Holland, J. M., Kao, J. Y., and Whitaker, W. J. (1984). A multi-sample apparatus for kinetic evaluation of skin penetration in vitro: The influence of variability and metabolic status of the skin. *Toxicol. Appl. Pharmacol.* **72**, 272-280.

BRRC Project 92N1108
Protocol Amendment 2
Page 9
Attachment 1

Study Design for *In Vitro* Skin Penetration Comparison of Excised Pig and Human Skin Samples

Phase I

Experiment	Number of Human & Pig Skin Samples	Description
1. Definitive Pig Skin Investigation	3 female pigs, 2 preparations/pig	Definitive study with ^{14}C -Mannitol and ^3H -Water.

Phase II

MLC Validation Work - Methods for 2 dye formulations to be conducted in Phase III.

Phase III

Experiment	Number of Human Skin Samples	Description
2. First Human Skin Sample	6 skin preparations per human sample	Evaluation of DB-79, Reactive Blue 19, and Direct Blue 218 dye formulations
3. Second Human Skin Sample	6 skin preparations per human sample	Evaluation of DB-79, Reactive Blue 19, and Direct Blue 218 dye formulations
4. Third Human Skin Sample	6 skin preparations per human sample	Evaluation of DB-79, Reactive Blue 19, and Direct Blue 218 dye formulations

Note: In Phase III experiments, all dye formulations and the ^{14}C -Mannitol standard and ^3H -Water will be done in duplicate.