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Dear Sir or Madam:

Enclosed please find a copy of the In Vitro Percutaneous Absorption Study conducted on 2-((4-amino-3-methylphenyl)ethylamino)ethanol sulfate (EPA Document Control Number 8EHQ-1185-0575). This report is being submitted as a follow up to our submission of November 11, 1985 on this compound. We are currently evaluating the need to update our Material Safety Data Sheet (MSDS). If you have questions concerning this report, please contact me at the address below.

Sincerely,

R. Hays Bell

R. Hays Bell, Ph.D, Director
Health and Environment Laboratories

RHB:JAF
Enc.

In Vitro Percutaneous Absorption Studies with
CD-4 Developer Preparations

D. Guest, Ph.D., Louise G. Perry and N.M. Teetsel, B.S.

Biochemical Toxicology Group
Toxicological Sciences Section
Health and Environment Laboratories

Eastman Kodak Company

June 24, 1987

PREFACE

This report presents data compiled during the investigation of the test compound specified. The sponsor, test compound, study type, test species, study dates and personnel responsible for the study are detailed in the Appendices. All raw data, standard operating procedures, protocols and other documents pertaining to this investigation are maintained in archival storage in the Toxicological Sciences Section, Health and Environment Laboratories, Eastman Kodak Company, Rochester, NY. Unless noted otherwise, all aspects of this study were performed in accordance with Good Laboratory Practice (GLP) Regulations. There were no known deviations from the GLP Regulations that significantly affected the quality of this study.

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ABSTRACT

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CD-4 (4-(N-ethyl-N-2-hydroxyethyl)-2-methylphenylenediamine sulfate) is used in photographic developing solutions. This study was designed to determine the absorption rate of CD-4 in aqueous solution, and in a commercial developing agent (Kodak Flexicolor® Color Developer) through rat skin *in vitro*, using Franz-type glass diffusion cells. For each experiment, four full-thickness skin samples were taken from each of 2 male Sprague-Dawley [CD®-(SD)BR] rats immediately prior to starting the first day of the study. The integrity of each skin sample was determined by measuring its permeability to tritiated water ($^3\text{H}_2\text{O}$). Two studies were performed; the first to examine the absorption rate of CD-4 in aqueous solution, and the second to determine the absorption rate of CD-4 in Kodak Flexicolor® Color Developer. Each study consisted of duplicate 3-day experiments, in which the permeability to $^3\text{H}_2\text{O}$ was determined on Days 1 and 3, and the permeability to CD-4 or CD-4 in Flexicolor® was determined on Day 2. The rate of increase in the concentration of $^3\text{H}_2\text{O}$ or CD-4 in the receptor chamber of each cell was used to calculate a permeability constant ($\text{cm}\cdot\text{hr}^{-1}$) and an absorption rate ($\text{mg}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ or $\mu\text{g}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$) for each material. The mean permeability constant for CD-4 in aqueous solution was $20.8 \times 10^{-3} \text{ cm}\cdot\text{hr}^{-1}$ ($\pm 7.27 \times 10^{-3}$, SEM). This was equivalent to a mean absorption rate of $93.7 \mu\text{g}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ ($\pm 32.7 \mu\text{g}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$, SEM). The mean permeability constant for $^3\text{H}_2\text{O}$ prior to exposure to CD-4 in aqueous solution was $3.37 \times 10^{-3} \text{ cm}\cdot\text{hr}^{-1}$ ($\pm 0.54 \times 10^{-3}$, SEM). The mean permeability constant for $^3\text{H}_2\text{O}$ on Day 3 after exposure to CD-4 in aqueous solution was $5.04 \times 10^{-3} \text{ cm}\cdot\text{hr}^{-1}$ ($\pm 1.24 \times 10^{-3}$, SEM). The mean permeability constant for $^3\text{H}_2\text{O}$ on Day 3 for control cells (not exposed to CD-4 in aqueous solution) was $5.10 \times 10^{-3} \text{ cm}\cdot\text{hr}^{-1}$ ($\pm 1.57 \times 10^{-3}$, SEM). The mean permeability constant for CD-4 in Flexicolor® was $102.9 \times 10^{-3} \text{ cm}\cdot\text{hr}^{-1}$ ($\pm 6.57 \times 10^{-3}$, SEM). This was equivalent to a mean absorption rate of $463.1 \mu\text{g}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ ($\pm 29.6 \mu\text{g}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$, SEM). The mean permeability constant for $^3\text{H}_2\text{O}$ prior to exposure to CD-4 in Flexicolor® was $2.76 \times 10^{-3} \text{ cm}\cdot\text{hr}^{-1}$ ($\pm 0.44 \times 10^{-3}$, SEM). The mean permeability constant for $^3\text{H}_2\text{O}$ on Day 3 after exposure to CD-4 in Flexicolor® was $9.75 \times 10^{-3} \text{ cm}\cdot\text{hr}^{-1}$ ($\pm 1.18 \times 10^{-3}$, SEM). The mean permeability constant for $^3\text{H}_2\text{O}$ on Day 3 for control cells (not exposed to CD-4 in Flexicolor®) was $1.56 \times 10^{-3} \text{ cm}\cdot\text{hr}^{-1}$ ($\pm 0.35 \times 10^{-3}$, SEM). Thus, the permeability of rat skin was significantly greater to CD-4 in Flexicolor® Color Developer than to CD-4 in aqueous solution alone. Exposure to CD-4 in aqueous solution had no significant effect on the permeability of rat skin to water, while CD-4 in Flexicolor® Color Developer caused a slight, but significant, increase in the damage ratio. These experiments allow the estimation of CD-4 uptake after skin exposure in humans, although the value obtained will probably overestimate the potential absorption of CD-4 in man, since, for a number of chemicals, human skin has been shown to be markedly less permeable than rodent skin. Using the absorption rates obtained for rat skin, and assuming that the body surface area of a 70 kg man 180 cm (71 inches) in height is about 1.85 m^2 , immersion of both hands (which comprise about 4% of the body surface area) in Flexicolor® Color Developer for one hour would result in the absorption of about 342.7 mg of CD-4, or about 4.9 mg/kg. Immersion of both hands in an aqueous solution of CD-4 for one hour would result in the absorption of about 69.3 mg of CD-4, or about 1.0 mg/kg.

Introduction

CD-4 (4-(N-ethyl-N-2-hydroxyethyl)-2-methylphenylenediamine sulfate, also referred to as 2-(4-amino-N-ethyl-m-toluidino)ethanol sulfate) is used in photographic developing solutions.

CD-4 has been tested for acute toxicity, skin and eye irritation, and for skin sensitization. The chemical was a moderate skin irritant on single application to guinea-pigs and this effect was exacerbated after repeated application.[1] CD-4 showed a strong reaction in a guinea-pig footpad sensitization assay and was a moderate irritant to rabbit eyes.[1] The results of the dermal acute toxicity study suggested that skin absorption was not occurring rapidly, since the LD₅₀ for male and female guinea-pigs was > 2000 mg/kg.[1] An oral gavage acute toxicity study in rats determined that CD-4 was moderately to highly toxic, and that females (LD₅₀ 35 mg/kg) might be more susceptible than males (LD₅₀ 81 mg/kg). Deaths were seen in males and females at doses of 25 mg/kg; no deaths were seen in rats receiving 12.5 mg/kg. The primary target organ was the kidney.[2]

In a four-week oral toxicity study, CD-4 was administered to male and female rats at doses of 10, 1 or 0.1 mg/kg/day. Toxicity was observed in the females receiving the highest dose. Toxic effects were restricted to renal tubular damage. No adverse effects were seen in males, or in the females dosed with 1 or 0.1 mg/kg/day.[3]

This study was designed to determine the absorption rate of CD-4 through rat skin in vitro and to assess the damage caused by the contact with the skin of the chemical contained in aqueous solution or in a commercial color developer solution, Kodak Flexicolor® Color Developer. For a large number of compounds, good agreement has been found between the results of in vivo and in vitro skin permeability studies.[4-6] The procedure followed in this study employed Franz-type glass diffusion cells, in which the skin sample formed a membrane between two chambers, one of which contained an excess of the test material in solution, and another which acted as a receptor for the chemical after skin penetration.

Materials and Methods

Materials

Franz-type diffusion cells (Figure 1) were obtained from Crown Glass Company (Somerville, NJ). The internal volume of the receptor chamber was determined and a calibration mark was made on the side arm as a constant volume indicator. Eight cells were housed in a cell drive unit/mounting assembly (Crown Glass Company, see Figure 1). The solution in the receptor chamber was stirred with a magnetic stirring bar and maintained at a constant temperature by the circulation of water at 37 °C.

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Chemicals

4-(N-ethyl-N-2-hydroxyethyl)-2-methylphenylenediamine sulfate, CD-4, (E.K. Accession No. 904984, HAEL No. 86-0039, CAS No. 25646-77-9) was obtained from Eastman Kodak Company, and Flexicolor® Color Developer, HAEL No. 86-0061, was purchased from a local photographic supply retailer (SRI, No. P7-86). The E.K. Accession numbers and CAS numbers for the principal components of Parts A, B and C of Kodak Flexicolor® Color Developer are listed below.

Part	Principal Component(s)	E.K. Accession No.	CAS Number
A	potassium carbonate	900409	584-08-7
	sodium metabisulfite	903498	7681-57-4
B	hydroxylamine sulfate	900150	10039-54-0
C	4-(N-ethyl-N-2-hydroxy-ethyl)-2-methylphenylenediamine sulfate (CD-4)	904984	25646-77-9

Structural confirmation and chemical purity of the CD-4 was obtained by HPLC/mass spectrometry (Appendix, p2). Prior to use, CD-4 was dissolved in deionized, distilled water and the pH of the solution was adjusted to about 10.5 (the nominal pH of Flexicolor® Color Developer solution).

Part C of the Flexicolor® Color Developer (containing CD-4) was analyzed by HPLC/MS for purity and structural confirmation (Appendix, p2). Prior to use, the developer solution was prepared as directed in the instructions provided with the product.

Animals

Male Sprague-Dawley [CD®-(SD)BR] rats were obtained from Charles River Breeding Laboratories, Wilmington, MA and were held in isolation for at least 5 days prior to use. Each rat was identified with a uniquely numbered ear tag. Water and food (Agway Prolab Animal Diet RMH 3000 Certified Pellets) was available ad libitum until time of sacrifice. At the time of sacrifice, the rats weighed 212 - 244g.

Collection of Skin Samples

Full thickness skin samples were taken immediately prior to starting the first day of the study. For each experiment, 4 skin samples were taken from each of 2 animals. The integrity of each

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skin sample was determined by measuring its permeability to tritiated water ($^3\text{H}_2\text{O}$, approximately 1 μCi per skin sample). On the first day, the test animals were euthanatized using CO_2 and the abdominal surface shaved carefully. The abdominal skin was removed from the animals and trimmed of excess fat and muscle tissue. An appropriate sized piece of skin was placed over the opening of the receptor chamber of each absorption cell. A PTFE O-ring was placed over the skin and the donor chamber was clamped in place to secure the skin.

Experimental Design

The study consisted of duplicate 3-day experiments, as follows:

- Day 1: determination of the permeability to $^3\text{H}_2\text{O}$;
- Day 2: determination of the permeability to CD-4 or Flexicolor[®] Color Developer;
- Day 3: determination of the permeability to $^3\text{H}_2\text{O}$.

A total of eight (8) cells was used for each experiment, consisting of one control cell and three test cells from each rat. The donor chamber of each cell designated as a control contained saline on Day 2, rather than CD-4 or Flexicolor[®] Color Developer.

On each day the receptor chambers were filled with isotonic saline containing an antibiotic-antimycotic solution (Penicillin, Amphotericin B and Streptomycin, Sigma Chemical Company, St. Louis, MO). On Day 2, the receptor solution also contained ascorbic acid (1 mg/mL) to inhibit oxidative degradation of absorbed CD-4. Adoption of this procedure was based on a preliminary study that established that the presence of ascorbic acid in the receptor solution did not affect the permeability of rat skin to $^3\text{H}_2\text{O}$ (see Appendix). Duplicate background (0 hour) samples (0.05 mL) were taken from each receptor chamber using the sampling port (Figure 1).

The donor solution ($^3\text{H}_2\text{O}$, CD-4 or Flexicolor[®] Color Developer) was applied in excess, to ensure steady state absorption kinetics. Duplicate samples were taken hourly from the receptor chambers for up to 8 hours and assayed either for radioactivity (by liquid scintillation spectrometry, LSS) after application of $^3\text{H}_2\text{O}$ on Days 1 and 3, or for CD-4 (by hplc) after application of CD-4 or Flexicolor[®] Color Developer on Day 2. The receptor chambers were refilled with the appropriate antibiotic-antimycotic solution after each sampling. After collection of the 8 hour samples on Days 1 and 2, the donor and receptor chambers were rinsed with saline, the donor chambers filled with saline and the receptor chambers filled with antibiotic-antimycotic mixture in saline. The cells were allowed to run overnight, emptied, rinsed and refilled with the appropriate solution for the day's experiment.

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The rate of increase in the concentration of radioactivity in the receptor chamber of each cell was used to calculate a permeability constant (cm.hr⁻¹) and an absorption rate (µg.cm⁻².hr⁻¹ or mg.cm⁻².hr⁻¹) for ³H₂O, CD-4 or Flexicolor® Color Developer.

Calculation of the Permeability Constant

The permeability constant and absorption rate were calculated for each cell using the slope of the linear plot of "concentration of material in the receptor chamber versus time" (Figure 2). The following equations were used to derive the permeability constants and absorption rates, respectively:

$$\begin{array}{l} \text{Permeability} \\ \text{Constant} \\ (\text{cm.hr}^{-1}) \end{array} = \frac{\text{slope(a)}}{\text{donor concentration x skin area (cm}^2\text{)}}$$

$$\begin{array}{l} \text{Absorption} \\ \text{Rate} \\ (\mu\text{g.cm}^{-2}\text{.hr}^{-1}) \\ \text{or (mg.cm}^{-2}\text{.hr}^{-1}) \end{array} = \frac{\text{slope(a)}}{\text{skin area (cm}^2\text{)}}$$

(a) slope from linear regression analysis of rate data, units of concentration per hour.

The permeability of each skin sample to ³H₂O was determined a second time, on the day following exposure to CD-4 or Flexicolor® Color Developer. The rate of ³H₂O permeability after exposure to CD-4 or Flexicolor® Color Developer (Day 3) and that obtained prior to exposure to CD-4 or Flexicolor® Color Developer (Day 1) was used to derive a 'damage ratio' function as follows:

$$\text{Damage Ratio} = \frac{\text{Permeability constant after exposure}}{\text{Permeability constant before exposure}}$$

This value provided an assessment of any alteration to the epidermal membrane of the skin resulting from contact with CD-4 or Flexicolor® Color Developer.

Statistical Analysis

The data from this study were examined using a computerized data analysis system [7] which employed descriptive statistics, normal plots, histograms, tests for outliers, Nested ANOVA, least squares

means and the probability differences between two alternatives. Outliers were identified and removed from further analysis. A statistically significant difference was detected using α of 0.01 for outliers and α of 0.05 for ANOVA and probability differences. The effects examined were differences between rats, replicate studies and treatment groups for each test material. In addition, differences were examined between these parameters for CD-4 and Flexicolor® Color Developer containing CD-4.

Results

The permeability data for each cell are shown in Tables 1 and 2, along with the overall mean permeability constants and absorption rates for CD-4 in aqueous solution and CD-4 contained in Flexicolor® Color Developer. All raw data pertaining to the study are presented in the Appendix.

A preliminary study established that addition of ascorbic acid (to inhibit oxidation of CD-4 in solution) had no effect on the permeability of rat skin to $^3\text{H}_2\text{O}$ (see Appendix, p8).

The mean permeability constant for CD-4 in aqueous solution was $20.8 \times 10^{-3} \text{ cm.hr}^{-1}$ (± 7.27 , SEM). This was equivalent to a mean absorption rate of $93.7 \mu\text{g.cm}^{-2}.\text{hr}^{-1}$ (± 32.7 , SEM). The mean permeability constant for CD-4 in Flexicolor® Color Developer was $102.9 \times 10^{-3} \text{ cm.hr}^{-1}$ (± 6.57 , SEM). This was equivalent to a mean absorption rate of $463.1 \mu\text{g.cm}^{-2}.\text{hr}^{-1}$ (± 29.6 , SEM), significantly greater than the rate for CD-4 alone ($\alpha < 0.05$).

The mean permeability constant for $^3\text{H}_2\text{O}$ prior to exposure to CD-4 in aqueous solution was $3.37 \times 10^{-3} \text{ cm.hr}^{-1}$ (± 0.54 , SEM). The mean permeability constant for $^3\text{H}_2\text{O}$ on Day 3 for control cells (not exposed to CD-4 in aqueous solution) was $5.10 \times 10^{-3} \text{ cm.hr}^{-1}$ (± 1.57 , SEM), not significantly different from the initial permeability constant for water. The mean permeability constant for $^3\text{H}_2\text{O}$ on Day 3 after exposure to CD-4 in aqueous solution was $5.04 \times 10^{-3} \text{ cm.hr}^{-1}$ (± 1.24 , SEM), also not significantly different from the permeability on Day 1. The mean permeability constant for $^3\text{H}_2\text{O}$ prior to exposure to CD-4 in Flexicolor® Color Developer was $2.76 \times 10^{-3} \text{ cm.hr}^{-1}$ (± 0.44 , SEM). The mean permeability constant for $^3\text{H}_2\text{O}$ on Day 3 for control cells (not exposed to CD-4 in Flexicolor® Color Developer) was $1.56 \times 10^{-3} \text{ cm.hr}^{-1}$ (± 0.35 , SEM), not significantly different from the initial permeability constant for water. The mean permeability constant for $^3\text{H}_2\text{O}$ on Day 3 after exposure to CD-4 in Flexicolor® Color Developer was $9.75 \times 10^{-3} \text{ cm.hr}^{-1}$ (± 1.18 , SEM). This value was significantly greater than the permeability constant for water on Day 1. Correspondingly, the damage ratio for CD-4 alone (1.72 ± 0.16) was not significantly different from the concurrent control value (1.17 ± 0.06), while

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the value for Flexicolor® Color Developer (3.00 ± 0.24) was slightly, but significantly greater than that of CD-4 in aqueous solution and that of the concurrent control group (1.11 ± 0.05).

Discussion

The rates of absorption through rat skin were determined in vitro for CD-4 in aqueous solution and CD-4 in Kodak Flexicolor® Color Developer. In both experiments, CD-4 was absorbed slowly, with absorption rates of 93.7 and $463.1 \mu\text{g}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ for CD-4 in aqueous solution and CD-4 in Kodak Flexicolor® Color Developer, respectively. The increased rate of absorption of CD-4 from Kodak Flexicolor® Color Developer compared to the rate of its absorption when dissolved in water is presumably a reflection of the effect of the other components of the developer solution on skin integrity. This suggestion is supported by the observation that the damage ratio for skin exposed to Kodak Flexicolor® Color Developer was slightly, but significantly greater than that for skin exposed to CD-4 in aqueous solution.

The absorption rates determined in this study probably overestimate human skin absorption rates, since rat skin has been shown to be considerably more permeable than human skin to a number of organic chemicals[8]. For example, the absorption rate reported for diethylene glycol monobutyl ether through human skin was $0.035 \text{ mg}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ [9] compared with a rate of $0.51 \text{ mg}\cdot\text{cm}^{-2}\cdot\text{hr}^{-1}$ for rat skin[10], different by a factor of almost 15. Skin from many experimental animal models has been found to be more permeable than that of man. For example, in vivo studies with humans and dogs exposed dermally to methyl *n*-butyl ketone demonstrated that humans absorbed between 16 and 27 mg of the chemical, compared with 77 mg in dogs.[11]

These experiments allow the estimation of CD-4 uptake after skin exposure in humans, making the following assumptions:

- 1) The body surface area of a 70 kg man 180 cm (71 inches) in height is about 1.85 m^2 :[12]
- 2) The surface area of the hands comprises about 4% of the body surface area (740 cm^2).

Using the percutaneous absorption rates determined in the rat, if both hands were immersed in an aqueous solution containing CD-4 for one hour, the total amount of chemical absorbed would be about 69.3 mg or about 1 mg/kg.

If both hands were immersed in a solution of Kodak Flexicolor® Color Developer containing CD-4 for one hour, the total amount of chemical absorbed would be about 342.7 mg or about 4.9 mg/kg.