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IN VITRO AND IN VIVO MEASUREMENT OF PERCUTANEOUS PENETRATION OF LOW MOLECULAR WEIGHT TOXINS OF MILITARY INTEREST  
ANNUAL/FINAL REPORT

AD-B151 419

B. W. Kemppainen; M. Mehta; R. G. Stafford;  
R. T. Riley; C. R. Clark

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19. ABSTRACT (Continue on reverse if necessary and identify by block number) The purpose of this contract is to: (i) use an <u>in vitro</u> system to assess the ability of low molecular weight toxins to penetrate through human and guinea pig skin, (ii) validate the results obtained in the <u>in vitro</u> system by conducting comparative studies of <u>in vitro</u> and <u>in vivo</u> cutaneous penetration in guinea pigs, (iii) assess the effects of specific solvent vehicles on the cutaneous penetration of the low molecular weight toxins, and (iv) modify the <u>in vitro</u> experimental system for skin penetration for use in studies of <u>in vitro</u> penetration across mucosal membranes. Toxins to be studied include the brevetoxins, microcystins, lyngbyatoxin A and debromoaplysiatoxin. Progress made during Years 1-4 of the contract is summarized below.			
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*25* \*Toxins and antitoxins, \*skin (anatomy)

*Molecular weight,*

19. Abstract Continued

- I. The penetration and distribution of [<sup>3</sup>H]PbTx-3 into pig skin was compared using in vivo and in vitro methods. The dose used in each topical study was 0.3 to 0.4 µg/cm<sup>2</sup> skin, with dimethylsulfoxide (DMSO) as the vehicle. In the in vivo study, mean cutaneous absorption after 48 hr (expressed as percentage of the dose) was 11.5% (n=3). In the in vitro study, mean cutaneous absorption after 48 hr was 1.6% (n=12), when based on accumulation of radioactivity in receptor fluid, or 9.9% when based on receptor fluid and dermis. [<sup>3</sup>H]PbTx-3 readily penetrated through the epidermis into the dermis, reaching maximal dermal accumulation at 4 hr (9.1% in vivo and 18% in vitro). At 24 hr, the amount in the dermis decreased to 2.3% and 15% in vivo and in vitro, and at 48 hr the amount in the dermis decreased to 8.2% in vitro. These results demonstrate the important role of the dermis as a reservoir for a lipophilic compound in both in vivo and in vitro percutaneous absorption studies.
  
- II. The purpose of this study was to determine the effect of vehicle on the penetration and distribution of brevetoxin ([<sup>3</sup>H]PbTx-3) into skin layers and receptor fluid. Disks of guinea pig skin were mounted on diffusion cells. The receptor fluid which bathed the dermal surfaces was Hanks' Balanced Salt Solution with gentamicin. The epidermal surfaces were dosed with 320 ng/cm<sup>2</sup> of PbTx-3 dissolved in 50 µl of vehicle (water, methanol, or dimethylsulfoxide [DMSO]), and exposed to ambient conditions. The diffusion cells were incubated for various time periods (0.25 - 48 hr) and distribution of radioactivity into skin layers and receptor fluid determined. Microtomed skin sections were extracted with methanol and radioactivity in each extract was measured with liquid scintillation counting. Regression models were fitted to the results for each compartment (skin surface, stratum corneum, epidermis, dermis, receptor fluid) and each treatment (vehicles). There was a significant relationship between accumulation of radioactivity in 3 separate compartments (epidermis, dermis and receptor fluid) and time when the vehicle was water or DMSO. In vitro skin penetration by PbTx-3 during 48 hr of exposure (calculated by summing radioactivity recovered in the dermis and receptor fluid) was 6.9, 4.7 and 15 for water, methanol, and DMSO, respectively (expressed as % of dose).
  
- III. The permeability coefficients (Kp) for THO were determined in human and monkey skin, and monkey buccal mucosa. Kp of human skin (0.47 X 10<sup>-3</sup> cm/hr) correlated favorably with the previous reports. Kp of hydrated monkey skin for THO (0.77 X 10<sup>-3</sup> cm/hr) was not significantly different (P > 0.05) for Kp of hydrated human skin (0.88 X 10<sup>-3</sup> cm/hr). Kp of monkey buccal mucosa for THO (6.15 X 10<sup>-3</sup> cm/hr) was significantly greater than that for monkey skin. Penetration and disposition of [<sup>3</sup>H]PbTx-3 into layers of monkey buccal mucosa and skin was determined. [<sup>3</sup>H]PbTx-3 (5-7 uCi) dissolved in 2 ml of water was applied to epithelial/epidermal surface (2.8 cm<sup>2</sup>) at zero time. The relative percent dose recovered from the upper layers of buccal mucosa (epithelium) and skin (epidermis) varied, but at each time interval was less than 2.5 percent of the dose. At most of the time intervals (2 to 24 hr), a larger percent of the dose was recovered from the inner layer of buccal mucosa (lamina propria) than from the inner layer of skin (dermis). After 24 hr, as much as 34 or 13 percent of the dose was

recovered from lamina propria or dermis, respectively. At each time interval studied, less than 2 percent of the dose of [<sup>3</sup>H]PbTx-3 penetrated into the receptor fluid which bathed the inner surfaces of the lamina propria and dermis. The results of this study demonstrate that monkey buccal mucosa is more permeable than skin to THO and PbTx-3.

- IV. Static diffusion cells were used to study the penetration of lyngbyatoxin A through excised guinea pig and human abdominal skin. The dermis was bathed in HEPES buffered Hanks' Balanced Salt Solution with gentamicin sulfate. The epidermis was dosed with 50 µg of lyngbyatoxin dissolved in 25 µl of DMSO. The cells were incubated at 37°C for varying time periods. High performance liquid chromatography was used to quantitate lyngbyatoxin A. The amount of lyngbyatoxin A recovered in the dermis and receptor fluid were summed for each time period studied. Previous studies have found that summing the amounts of a compound found in the dermis and receptor fluid correlates well with *in vivo* skin absorption. Statistical analysis showed that there were no significant differences between the different time periods for the guinea pig or the human skin penetration data. T-tests performed to compare the human and the guinea pig skin penetration showed statistically significant difference ( $p \leq 0.05$ ) between each mean at each time period. The amounts of lyngbyatoxin A present in the dermis and receptor fluid of the guinea pig were:  $25 \pm 7.4\%$ ,  $25 \pm 4.6\%$ ,  $29 \pm 3.3\%$  and  $23 \pm 3.0\%$  (mean  $\pm$  SE) at the 1, 4, 24 and 48 hr time periods, respectively. The amounts in the dermis and receptor fluid of the human were:  $5.7 \pm 1.3\%$ ,  $5.2 \pm 1.1\%$ , and  $7.2 \pm 1.4\%$  at the 1, 4, and 24 hr time periods, respectively. Skin penetration of lyngbyatoxin A in the guinea pig was 4.3, 4.9, and 3.9 times more than in the human at 1, 4, and 24 hr. These results indicate that lyngbyatoxin readily penetrates into excised guinea pig and human skin.
- V. The percutaneous penetration of microcystin was compared through excised skin discs of mouse, guinea pig and human abdominal skin. The rate of percutaneous penetration was determined by high pressure liquid chromatography (HPLC) by measuring the accumulation of microcystin in the skin and receptor fluid which bathed the dermal side of excised skin. The effect of vehicle [water, dimethylsulfoxide (DMSO) and methanol] on penetration of microcystin through human skin was also evaluated. When DMSO was the vehicle, the microcystin penetration through the excised discs of skin after 48 hr (expressed as percent of dose,  $36 \mu\text{g}/\text{cm}^2$ ), was  $3.2 \pm 1.0$ ,  $1.4 \pm 0.7$  and  $1.8 \pm 0.7$  (mean  $\pm$  SD) for mouse, guinea pig and human skin, respectively. The percent of dose which penetrated through the human skin was  $0.9 \pm 0.3$  and  $3.8 \pm 0.06$  when water or methanol was the vehicle, respectively. The comparison of 3 different doses, 3.2, 7.2 and  $32 \mu\text{g}/\text{cm}^2$  (vehicle, DMSO) demonstrated that total amount which penetrated increased as the dose increased, but the percent of dose that penetrated significantly decreased at the higher doses. This study indicated that a very small amount of microcystin penetrated the excised human skin and that guinea pig skin was a better model than mouse for the study of penetration of microcystin through human skin.

## FOREWORD

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METHODS FOR IN VITRO SKIN ABSORPTION STUDIES OF A LIPOPHILIC  
TOXIN PRODUCED BY RED TIDE

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STATEMENT OF PROBLEM

It is not known if brevetoxins penetrate skin in sufficient concentration to cause systemic effects. The purpose of this study was three fold. Firstly, to use in vitro methods to assess the skin absorption of a lipophilic red tide toxin and the potential for systemic toxicity. Secondly, to evaluate in vitro methodologies for assessing skin penetration by a lipophilic compound. Thirdly, to determine if the dermis functions as a reservoir for a topically absorbed lipophilic compound.

BACKGROUND

PbTx-3 is one of a group of brevetoxins produced by the marine algae, Ptychodiscus brevis. Blooms of this algae are referred to as red tide and occur off the coasts of Florida (1). People swimming during 'red tides' sometimes experience local dermal toxic effects which include skin irritation and itching (2). These observations indicate that brevetoxins can penetrate

at least through the layers of the stratum corneum and into the viable epidermis to cause toxic effects.

#### RATIONALE USED IN CURRENT STUDY

Dimethylsulfoxide (DMSO) was used as the vehicle for the topical studies in order to determine the amount of skin penetration during a worst case scenario, e.g., if a laboratory worker was extracting brevetoxin from cultured algae and spilled brevetoxin dissolved in a solvent on his or her skin. Weanling pig skin was used as a model for human skin because it has similar permeability properties (3).

#### EXPERIMENTAL METHODS

##### MATERIALS

Unlabeled PbTx-3 and labeled (42-<sup>3</sup>H)PbTx-3 (see Figure 1 for chemical structure) were obtained from Dr. Daniel Baden, University of Miami, Miami, FL. The radiolabeled (42-<sup>3</sup>H)PbTx-3 had a specific activity of 12 to 14 Ci/mmol. For some of the topical studies, the specific activity of the [<sup>3</sup>H]PbTx-3 was decreased to 0.9 to 1.8 Ci/mmol by adding unlabeled PbTx-3. The radiochemical purity was determined prior to each experiment, using high performance liquid chromatography (HPLC). The HPLC methods involved using a uBondapak C18 reverse phase column (Waters, Milford, MA), a mobile phase of methanol:water (85:15, v/v), a flow rate of 0.5 ml/min, a monitoring of ultraviolet light absorbance at 215 nm and a monitoring of radioactivity with a FLO-ONE radioactive flow detector (Radiomatic, Tampa, FL). The retention time ( $R_t$ ) of [<sup>3</sup>H]PbTx-3

was 4.0 to 4.2 min. The radiochemical purity was determined to be greater than 98%. The contaminant peak had a  $R_f$  of 1.0 to 1.3 min. Previous studies have shown that the [ $^3\text{H}$ ]PbTx-3 is stable in DMSO and methanol at 37°C for at least 48 hr (4).

### Animals

Weanling pigs weighing between 9 and 17 kg were used for the studies. Female Yorkshire pigs obtained from Boswell Laboratory Animals (Corcoran, CA) were used to determine the in vivo percutaneous penetration of PbTx-3. Male and female Yorkshire-Duroc-Landrace cross-bred pigs obtained from Auburn University Swine Production Unit (Auburn, AL) were used to determine in vivo skin retention and in vitro skin penetration and retention. The skin penetration and retention of [ $^3\text{H}$ ]PbTx-3 in Yorkshire and cross-bred pig skin was compared using in vitro methods. The skin barrier properties of these two skin types were found to be similar (results not shown).

### In vivo percutaneous penetration of PbTx-3

This study was divided into two phases. Firstly, the disposition of radiolabel in urine, feces, liver, kidney, spleen and muscle was determined after subcutaneous administration of [ $^3\text{H}$ ]PbTx-3 to three pigs. Secondly, the disposition of radioactivity following topical administration of [ $^3\text{H}$ ]PbTx-3 to three additional pigs was determined. Percutaneous penetration was determined by dividing the amount (percent) of radioactivity excreted in the urine and feces after topical application by the

corresponding amount (percent) excreted after subcutaneous administration and multiplying the result by 100.

Radiolabeled PbTx-3 (1.1 ug, 18 uCi) dissolved in 0.5 ml propylene glycol was administered to three pigs subcutaneously. This dose was selected because it was small enough so as not to cause toxic effects in the pigs (5,6), but large enough to enable detection of the radiolabeled compound in urine, feces and tissue. The pigs were then housed in stainless steel metabolism cages for approximately 3 days. Urine and feces were collected twice a day. The pigs were then sedated by intramuscular injection of a mixture of xylazine HCl (3 mg/kg, Rompun, Miles Laboratories, Shawnee, KS) and ketamine HCl (20 mg/kg, Vetalar, Parke-Davis, Morris Plains, NJ), and euthanized by an intravenous injection of T-61 Euthanasia solution (American Hoechst Corp, Somerville, NJ). The liver, kidney, spleen and a portion skeletal of muscle (pectoralis) were removed from each pig. Radioactivity in urine samples was determined by mixing an aliquot (1 ml) of each urine sample with 10 ml scintillation cocktail (Optifluor, Packard Instrument Company, Downers Grove, IL). Radioactivity was measured with a Packard 1500 scintillation spectrophotometer fitted with an external standard. The feces were freeze-dried and samples placed in zip-lock polyethylene bags and ground to a fine powder with a teflon rolling pin. A 0.2 g aliquot was taken from each sample and mixed with 0.2 g of cellulose powder and oxidized in a Packard Model 306 Sample Oxidizer. Radioactivity in these vials was then determined by standard liquid scintillation counting (LSC). The liver, kidney, spleen and

portion of skeletal muscle were processed as described for fecal samples, and radioactivity was determined similarly. The percentage of the dose in the total skeletal muscle mass at death was calculated based on pig body weight and published values for the body composition of pigs 28 d postnatal (7).

Pigs were prepared for the topical studies by clipping the hair (Oster Model A-2, Milwaukee, WI) on the upper back and marking a square application site (5 cm x 5 cm). The dose (0.4 ug [3H]PbTx-3/3 ul DMSO/cm<sup>2</sup>, 20 uCi) was applied to the application site with a blunt tipped glass syringe (Hamilton, Reno, NV). A non-occlusive protective patch was constructed and placed over the application site as previously described (8). The patch kept the application site from contacting the metabolism cage and prevented radioactive exfoliated skin from contaminating urine and feces. The pigs were housed in metabolism cages for approximately 4 days and urine and feces collected as described above. On day 2 the protective patches were removed and replaced with new patches. On day 3 (48 hr post application) the patches were removed and the site of application decontaminated with cotton moistened with soap and water. The radioactivity in the patches and cotton swabs was recovered by extraction with methanol and quantified by LSC. Pigs were sedated by an intramuscular injection of acepromazine maleate (0.2 mg/kg, Ayerst Laboratories, New York, NY) and euthanized by an intravenous injection of T-61. The skin from the application site was removed and sectioned into epidermis (100 um), dermis (approximately 2000 um), and subcutaneous fat with an electric

dermatome (Brown Electrodermatome Model 901, Zimmer-USA, Warsaw, Ind.). As each successive layer was removed, the skin layer was placed in a scintillation vial which contained 10 ml of methanol. The dermatome cutting surface was decontaminated with methanol moistened cotton after each layer was cut. The cotton swab was then extracted with methanol and the amount of compound measured was added to that contained in the previous layer of skin. The dermis was removed with two passes of the dermatome set to cut a 1000 um layer. The remaining tissue consisted of subcutaneous fat. Skin layers were extracted by sonication in methanol for 10 min. An aliquot of the methanol was mixed with counting solution for LSC.

#### In vivo percutaneous retention of PbTx-3

Four pigs were prepared for this study by clipping the hair on their upper backs and marking four rectangular application sites (2 cm x 4 cm) on each pig. The dose (0.4 ug [<sup>3</sup>H]PbTx-3/3 ul DMSO/cm<sup>2</sup>, 3.1 uCi) was applied to each application site at 0.25, 1.0, 2.0, 4.0, or 24 hr prior to when the pigs were euthanized. The application sites were covered with a non-occlusive protective patch (see above for description). The pigs were housed in steel metabolism cages during the topical exposure period. The pigs were sedated with an intramuscular injection of a mixture of xylazine and ketamine (see above for dose), and euthanized by an intravenous injection of T-61. The protective patches were removed and skin from the upper back excised and cut into sections (6 cm x 12 cm), each of which contained one of the

application sites. The surface of each application site was decontaminated with cotton moistened with soap and water. The skin was sectioned into epidermis (100 um), dermis (approximated 2000 um) and subcutaneous fat. The skin sections and cotton swabs were each extracted with methanol and an aliquot of each extract was counted with LSC.

In vitro skin penetration and retention of PbTx-3: Determined with skin disks incubated in diffusion cells

Skin was excised from the upper back of female and male weanling pigs. Split-thickness skin consisting of the epidermis and a portion of the dermis (total thickness of 900 um) was prepared with a Padgett Electro Dermatome (Padgett Instruments, Kansas City, MO) and mounted on glass flow-through diffusion cells (Laboratory Glass Apparatus, Berkeley, CA). The diffusion cells have a water jacket which maintains the receptor fluid at 37°C. Each cell exposes 1 cm<sup>2</sup> of skin surface area and has a receptor fluid volume of 3 ml. Hepes-buffered Hanks' balanced salt solution (HHBSS, GIBCO, Grand Island, NY) was used as the receptor fluid which bathes the dermal surface of the skin disk. HHBSS has been shown to maintain the viability (as measured by glucose utilization) of excised skin in diffusion cells (9). The receptor fluid was pumped through the diffusion cells at the rate of approximately 5 ml per hour. The skin surfaces were each dosed with 0.3 ug [<sup>3</sup>H]PbTx-3/6 ul DMSO/cm<sup>2</sup> (5 uCi), using a Hamilton microliter syringe with a blunt tip. The topical dose applied to the excised skin mounted on diffusion cells was 25%

less than the topical dose ( $0.4 \text{ ug/cm}^2$ ) used in the in vivo study. This small difference in the size of the dose would not significantly alter percutaneous penetration when values are expressed as percent of dose. Increasing the amount of the applied dose results in an increase in the total dose absorbed, but the proportion of the dose absorbed (percent dose) is not significantly different when the dose is changed by less than 100% (10,11). The epidermal surfaces were exposed to ambient air ( $22^\circ\text{C}$ ) during the entire length of the experiment. Penetration of [ $^3\text{H}$ ]PbTx-3 into skin layers and receptor fluid was determined by incubating the diffusion cells for various time intervals (0.25, 1.0, 2.0, 4.0, 24, and 48 hr) and measuring radioactivity in skin layers and receptor fluid. Accumulated radioactivity (PbTx-3 and metabolites) in receptor fluid was determined by collecting receptor fluid at hourly intervals and analyzing radioactivity in each sample with standard LSC techniques. The epidermal surfaces were decontaminated with cotton moistened with soap and water to determine the amount of toxin remaining on the skin surface. The area of the skin dosed with toxin was cut away from the perimeter portion of the skin. The perimeter portion of the skin was extracted with methanol to determine if part of the applied dose had diffused to the outer portion of the skin disk and was not available for diffusion through the skin into the receptor fluid. The central portion of each skin disc was mounted on embedding blocks (Thomas Scientific, Swedesboro, NJ) with Tissue-Tek OCT Compound (American Scientific Products, Stone Mountain, GA), frozen, and sectioned parallel to the skin surface

with a microtome (Bausch and Lomb, Optical Co, Rochester, NY). The epidermis (100 um) and dermis (remaining 800 um) were each extracted twice with methanol. The radioactivity in receptor fluid, extracts of skin wash and skin sections was summed to determine total recovery of the applied dose.

In vitro skin penetration and retention of PbTx-3: Determined with skin slabs incubated on absorbent material moistened with tissue culture media

Full-thickness skin (including subcutaneous fat) was obtained from the upper back of two female weanling Yorkshire pigs. Rectangular pieces (6 cm x 12 cm) were placed dermal side down on stainless steel trays lined with gauze pads moistened with RPMI media (GIBCO). The 2 cm x 4 cm areas for toxin application were outlined with an alcohol marker. The toxin (0.4 ug [<sup>3</sup>H]PbTx-3/3 ul DMSO/cm<sup>2</sup>, 2.3 uCi) was applied to each application site. The dosed skin segments were divided into 4 groups which were incubated at room temperature (22°C) for different periods of time (0.25, 1.0, 2.0 and 4.0 hr). This in vitro experiment was conducted under different temperature conditions than the diffusion cell experiment (37°C) in order to determine if this simple, inexpensive procedure could replace the more complicated method of using temperature controlled diffusion cells. The distribution of radiolabeled toxin into layers of the skin was determined by first decontaminating the surface of the skin with cotton moistened with soap and water to recover [<sup>3</sup>H]PbTx-3 which remained on the skin surface. The skin was

layered into epidermis, dermis, and subcutaneous sections and extracted with methanol as described for in vivo skin retention studies. A portion of the methanol extracts were mixed with counting solution for LSC. The remaining portions of the methanol extracts were individually evaporated to dryness under a steady stream of nitrogen and immediately dissolved in 100 ul methanol. The radiochemical composition of the skin extracts was determined with HPLC (see first paragraph of EXPERIMENTAL METHODS section for HPLC method).

A control group was included in order to determine if radioactivity on the dermatome cutting surface was contaminating subsequent skin sections and thus confounding the results. This was done by placing skin segments dermal side down on absorbent material moistened with RPMI medium (as described above). The epidermal surfaces of skin in the treated group were dosed with 0.4 ug [<sup>3</sup>H]PbTx-3/3 ul DMSO/cm<sup>2</sup> and the skin segments in the other group were dosed only with the vehicle (DMSO). All of the skin segments were incubated for 45 min and the skin surfaces were decontaminated as described above. The dermatome was used to: (i)remove the epidermis of one of the skin segments which was dosed with DMSO only and (ii)remove the epidermis of one of the skin segments which had been dosed with [<sup>3</sup>H]PbTx-3. The dermatome cutting surface was decontaminated with cotton moistened with methanol and was used to remove a section of the dermis from the skin segment which had been dosed with DMSO only. This procedure was used to determine the amount of toxin which was carried over from slicing the epidermal layer (which had the

highest level of radioactivity) to the dermal layer.

The in vivo and in vitro percutaneous penetration and retention of [<sup>3</sup>H]PbTx-3 was statistically analyzed by one-way analysis of variance and the significant mean differences were estimated, using a Duncan's multiple range test (12).

## RESULTS

### In vivo percutaneous penetration of PbTx-3

The disposition of radioactivity following subcutaneous and topical application of [<sup>3</sup>H]PbTx-3 to weanling pigs is shown in Table 1. The mean percent dose cutaneously absorbed ( $11.5 \pm 1.8$ [SE]) during 48 hr of topical exposure was calculated by dividing the percent dose excreted following topical administration ( $2.3 \pm 0.3$ ) by the percent dose excreted following subcutaneous administration ( $20 \pm 6.9$ ), and multiplying by 100. Analysis of radioactivity in selected organs following subcutaneous administration indicated that muscle had the largest concentration, followed by liver, spleen and kidney. The total recovery of the dose was low (36% and 80%, following subcutaneous and topical administration, respectively). The low recovery rates were probably due to residual radioactivity in carcass components which were not analyzed. Thirty nine percent of the topical dose was recovered in the protective patches which were removed on day two. This indicated that normal exfoliation of skin resulted in removal of 39% of the topical dose. This amount of exfoliation is consistent with a previous report (3).

### In vivo percutaneous retention of PbTx-3

The disposition of radioactivity into skin layers following topical application of [<sup>3</sup>H]PbTx-3 to weanling pigs is shown in Figure 2. Accumulation of radioactivity in the epidermis was significantly ( $P < 0.05$ ) greater at 24 hr than at 0.25, 1, 2, or 4 hr. Accumulation of radioactivity in the dermis was not significantly different ( $P > 0.05$ ) at any of the time periods examined. Nine percent of the dose had penetrated into the dermis during the first four hours of exposure. Therefore, 78% of the dose cutaneously absorbed during 48 hr had penetrated into the dermis within the first 4 hr of exposure. At each time interval examined the amount of radioactivity in the epidermis was greater than the amount in the dermis. The total recoveries of the applied doses ranged from 61 to 94 percent. The radioactivity in the protective patches was not determined and this may be responsible for the low total recoveries.

### In vitro skin penetration and retention of PbTx-3: Determined with skin disks incubated in diffusion cells

The disposition of radioactivity into skin layers and receptor fluid following topical application of [<sup>3</sup>H]PbTx-3 to excised skin mounted on flow-through diffusion cells is shown in Figure 3. The accumulation of radioactivity in the epidermis was significantly greater at 24 and 48 hr than at 0.25, 2, or 4 hr. Accumulation of radioactivity in the dermis was significantly greater at 1 and 4 hr than at 0.25, 2, 24, or 48 hr. These significant differences at the various time periods indicate that

the system is dynamic and had not reached equilibrium at the earliest time periods. In contrast to in vivo disposition into skin layers, the amount of radioactivity in the dermis was greater than the amount in the epidermis at all time intervals which were studied except 48 hr. The mean percent dose which penetrated into the dermis and receptor fluid was  $8.2 \pm 0.81$  (SE) and  $1.6 \pm 0.50$  (respectively) after 48 hr of exposure. In vivo and in vitro percutaneous penetration of PbTx-3 (11.5 and 9.9 percent of the dose, respectively) were not significant ( $P > 0.05$ ) when the in vitro value was calculated by summing radioactivity in the dermis (8.2%) and receptor fluid (1.6%). However, in vitro skin penetration of PbTx-3 significantly ( $P < 0.01$ ) underestimated in vivo penetration when the in vitro value was based only on accumulation of radioactivity in receptor fluid. Recovery of radioactivity in the receptor fluid is indicated only at times 1.0, 24 and 48 hr, because it was not possible to indicate on this figure the very small levels of radioactivity (0 to 0.005 percent of the dose) in the receptor fluid at the other time intervals.

In vitro skin penetration and retention of PbTx-3: Determined with skin slabs incubated on absorbent material moistened with tissue culture media

The disposition of radioactivity into skin layers during topical exposure of excised skin slabs to [ $^3\text{H}$ ]PbTx-3 is shown in Figure 4. Accumulation of radioactivity in the epidermis was significantly greater at 2 and 4 hr than at 0.25 and 1 hr. The

amount of radioactivity in the dermis was not significantly different at any of the time periods examined. The accumulation of radioactivity in the epidermis and dermis was considerably less than that observed in the in vivo or in vitro diffusion cell experiments. Accumulation of radioactivity in the epidermis was greater than that in the dermis, which is consistent with the in vivo finding. However, the amount (percent of the dose) in the epidermis (3.5 to 7.2) and dermis (1.6 to 2.9) in the skin slab experiment was approximately half the amount in the epidermis (8.0 to 27) and dermis (2.7 to 9.1) in the in vivo study.

Results from the control study indicate that radioactivity remaining on the dermatome cutting surface after decontamination with methanol resulted in contamination of the dermis with 0.13 percent of the dose. In other words, less than 10% of the 2 to 3 percent of the dose extracted from the dermis was due to contamination of the dermatome cutting surface.

Analysis of the radiochemical composition of extracts of the skin washes and skin sections indicated that greater than 95% of the radioactivity in each sample was associated with PbTx-3.

#### DISCUSSION

The mean in vivo skin absorption of PbTx-3 was 11.5 percent of the dose when determined by correction for incomplete excretion of the radiolabel following topical absorption. The validity of the excretion method was tested by comparing the percutaneous penetration of nine xenobiotics in nude mice (3) when determined by the excretion method versus the mass balance

method. In the latter method percutaneous absorption is calculated by summing the radioactivity in the urine, feces, tissues and carcass (3). The significant correlation ( $r=0.97$ ,  $P=0.05$ ) between skin penetration when determined by the excretion method versus the mass balance method is an indication that the excretion method is a valid method for determining in vivo skin absorption. The low recovery of dose in the in vivo study reported here is a consequence of not being able to extract the entire carcass (as is done in rodents).

The underlying assumption when using the excretion method to calculate percutaneous absorption is that PbTx-3 or its metabolites are excreted similarly regardless of the route of administration. If this assumption is true, then the fecal fraction ([radioactivity in feces] divided by [radioactivity in urine and feces]) of radioactivity should remain the same after topical and parenteral administration. This assumption is true for PbTx-3, since the fecal fraction is 52% and 45% following topical and subcutaneous administration, respectively. The fecal fraction of radioactivity was also similar following topical versus subcutaneous administration for eight out of nine xenobiotics studied by Reifenrath et al., (3). However, Carver and Riviere reported a change in urinary-fecal excretion ratios following topical versus intravenous administration for five of the six compounds studied (13). The altered excretion patterns could be due to the larger dose ( $40 \text{ ug/cm}^2$ ) compared to the dose administered by Reifenrath et al., ( $4 \text{ ug/cm}^2$ ). Sanders et al., (14) found that the fecal fraction of excreted radioactivity

increased when the topical dose was increased.

The in vivo percutaneous absorption results for PbTx-3 have been used to calculate the dose absorbed during cutaneous exposure to PbTx-3 in a hypothetical research laboratory environment. The dose absorbed in the hypothetical laboratory environment was calculated by adjusting the percent dose absorbed in the in vivo study reported here, in regard to the exposed body surface area and duration of exposure. The hypothetical situation was that a laboratory worker spilled DMSO containing PbTx-3 on his hands. The worker's body weight is assumed to be 70 kg. The surface area of his hands (860 cm<sup>2</sup>) were assumed to be exposed under non-occluded conditions for one hour (15). The dose used for this calculation was the same as used in the in vivo study (0.4 ug PbTx-3/cm<sup>2</sup>). The following additional assumptions are made: i) percutaneous penetration in human hand will equal that in pig upper back, ii) extent of absorption in 48 hr will be proportional to absorption in 1 hr, iii) elimination will not significantly effect sustained dose, and iv) temperature and hydration are similar to that used in laboratory (16). The amount of absorption in the laboratory worker's hands (39.6 ug) is equivalent to 0.57 ug/kg. The dose of PbTx-3 which caused acute lethality in mice was 94 or 170 ug/kg when administered intravenously or intraperitoneally, respectively (5). Intravenous administration of 1.0 to 80 ug/kg caused bronchoconstriction in anesthetized guinea pigs (6). Cutaneous absorption of PbTx-3 in the hypothetical laboratory situation was 165 to 298 times less than the dose which caused lethality in

mice, and 1.8 to 140 time less than the dose which caused bronchoconstriction in the guinea pig. The minimum dose of PbTx-3 which caused systemic toxic effects in humans is not known. These results indicate that cutaneous exposure to high levels of PbTx-3 in the presence of penetration enhancers would lead to systemic effects. Topical application of PbTx-3 (3500 ug/kg) dissolved in 90% DMSO did not cause lethality in guinea pigs (17). The absence of systemic effects in guinea pigs following the large topical dose of PbTx-3 could be due to a slow rate of skin absorption coupled with extensive metabolism. Poli et al., (18) reported that PbTx-3 was extensively metabolized following intravenous administration to rats.

The percutaneous penetration of [<sup>3</sup>H]PbTx-3 during 48 hr of exposure has been compared using *in vivo* and *in vitro* (diffusion cells) methods. The large amount of variability in the *in vitro* studies is a well recognized characteristic of *in vitro* percutaneous penetration studies. Several methods have been suggested to help reduce this variability (19, 20, 21), but in general the large amount of variability is accepted as one of the characteristics of these types of studies.

PbTx-3 is a lipophilic, large molecular weight compound. The solubility of PbTx-3 in water is approximately 100 mg/l (Baden, personal communication). The molecular weight of PbTx-3 is 896 (22). *In vitro* methods are widely used to measure skin absorption of xenobiotics (23). In general these *in vitro* methods involve mounting disks of excised skin on diffusion cells, applying the xenobiotic to the epidermal surface and

quantifying accumulation of penetrant in an aqueous solution (receptor fluid) which bathes the dermal surface (24,25,26). The validity of using in vitro methods to measure percutaneous absorption has been evaluated by comparing in vivo and in vitro skin absorption of compounds of varying physical and chemical properties. The degree of correlation between in vivo and in vitro skin penetration depends on the compound and methodology (27,28). The ratio of in vivo to in vitro skin penetration increases for more lipid soluble compounds (24). It has been hypothesized that this discrepancy is due to limited solubility of the lipophilic penetrant in the hydrophilic receptor fluid (29). This hypothesis is substantiated by the observation that dermal accumulation of lipophilic penetrants is significantly greater in vitro than in vivo (30). An alternate explanation would be the poor diffusability of lipophilic or high molecular weight compounds in the dermis (31). This increased diffusional resistance in the dermis is exaggerated in the in vitro system due to the lack of functional vasculature to remove penetrant from dermal tissue. This alternate explanation is supported by reports that in vitro skin penetration by hydrophobic compounds is not altered (24) or minimally increased (29,32) when saline receptor fluid is replaced with serum. For example, in vitro skin penetration by the hydrophobic compound hexachlorophene was increased by a factor of two, even though the solubility of hexachlorophene in serum is 500 times greater than in water (32). Several modifications of in vitro methodology have been used in attempts to resolve the discrepancies between in vivo and in

vitro skin penetration of lipophilic compounds. One alternative method involves using surfactants or solvents as the receptor fluids (29,33). The disadvantage of using non-physiologic receptor fluid is that cutaneous metabolism is disrupted (9), which can in turn alter percutaneous penetration (25). Another alternative method is to measure dermal and receptor fluid levels of penetrant at different time intervals in order to approximate in vivo penetration (34). The results of this study support the validity of this latter method. Hawkins and Reifenrath (34) compared the in vitro and in vivo skin absorption of nine compounds which had octanol-water partition coefficients which ranged from 0.01 to 3.78. They found better agreement between in vitro and in vivo skin absorption when in vitro values were calculated by summing penetrant recovered from both the dermis and receptor fluid (34). Bronaugh et al., (35) and Wester et al., (36) have also reported that in vitro values obtained by combining penetrant recovered in the receptor fluid and skin contents resulted in a more accurate value for absorption. The consistent findings of these four separate reports indicate that the discrepancies between in vitro and in vivo percutaneous penetration can be minimized by calculating in vitro values by summing penetrant recovered in the dermis and receptor fluid.

The intra-cutaneous retention of [<sup>3</sup>H]PbTx-3 during these exposure periods was compared using an in vivo method and two in vitro methods. In all of these studies soap and water were used to remove the unabsorbed test compound. Soap and water are the materials most routinely used to decontaminate topically exposed

skin (35,37). It has been shown that soap and water increase the percutaneous penetration of some compounds (37). It is important that this method of decontamination was included in these studies since it is likely that a person who is accidentally exposed to PbTx-3 in a research laboratory would use soap and water to decontaminate his or her skin. If the soap and water decontamination procedure was the main factor responsible for the distribution of radioactivity in the skin layers, then the levels of radioactivity in the skin layers would be greatest when the amount of radioactivity on the skin surface was the greatest (at the earliest time period). However, the level of radioactivity in the dermis in the in vitro (diffusion cell) experiment was significantly greater ( $P < 0.05$ ) at 4 hr than at 0.25 hr. In both the in vivo and in vitro (diffusion cell) experiments the accumulation of [ $^3\text{H}$ ]PbTx-3 in the dermis reached a maximum level after 4 hr of exposure (9.1 and 18 percent dose of the dose, respectively). Formation of a dermal reservoir is contrary to the generally held belief that during percutaneous penetration most of the penetrant is absorbed by the capillary bed at the dermo-epidermal junction (29, 33, 38,39). The presence of a dermal reservoir is evidenced by other reports which have found relatively high levels of penetrant in the dermis following topical exposure (40,41).

The existence of a dermal reservoir in vivo and in vitro demonstrates the important role the dermis plays in percutaneous absorption. The hypothetical significance of a dermal reservoir is three fold. Firstly, in the case of lipophilic toxins the

increased residence time in the dermis would result in more severe local toxic effects and reduced systemic effects. This hypothesis is substantiated by an in vivo report by Pang et al., (42) in which a lipophilic toxin (T-2 toxin) and its metabolites were recovered from the skin and subcutaneous tissue at the site of topical application for up to 14 d after dosing. The necrotizing dermatitis was still present at the application site 14 d post T-2 toxin treatment; however, the injury to the other organs was mild. Secondly, in the case of topical application of lipophilic pharmaceutical agents, the increased residence time in the dermis would result in more effective treatment of cutaneous disease, accompanied by reduced systemic toxicity. Thirdly, the effect of a dermal reservoir on in vivo and in vitro percutaneous penetration of lipophilic compounds. As mentioned above, it is important to quantify the penetrant in the dermal reservoir when using in vitro methods to calculate percutaneous absorption.

After 24 hr of exposure, the in vivo dermal reservoir was reduced to 2.3 percent of the dose. This was probably due to removal of [<sup>3</sup>H]PbTx-3 from the dermis via dermal vasculature; and reduced penetration of [<sup>3</sup>H]PbTx-3 from epidermis into dermis due to diminished vehicle effects. The in vitro percutaneous penetration of PbTx-3 is enhanced by DMSO by factors of two or three when compared to when water or methanol was the vehicle (manuscript in preparation). The enhancement of percutaneous penetration by DMSO is temporary and reaches complete extinction three hr after treatment due to absorption of DMSO by circulation (43).

In the in vitro diffusion cell system there appears to be a "back diffusion" or exsorption of [<sup>3</sup>H]PbTx-3 during the time period of 4 to 48 hr. During this time the sum of the radioactivity in the dermis and receptor fluid of the in vitro diffusion cells had decreased from 18 to 10 percent of the dose. During the time period of 4 to 24 hr the radioactivity in the epidermis significantly ( $P < 0.05$ ) increased from 4.8 to 12 percent of the dose. This sequence of events suggests there is exsorption of [<sup>3</sup>H]PbTx-3. In vivo percutaneous exsorption of xenobiotics during topical exposure to DMSO has been reported (44).

The larger dermal reservoir observed in vitro than in vivo is consistent with previous reports (30,41) and is thought to be due to the absence of functional vasculature in vitro and limited solubility of lipophilic penetrants in aqueous receptor fluid. The penetration of [<sup>3</sup>H]PbTx-3 into epidermis, dermis and subcutaneous fat was considerably less for the in vitro skin slab method than in vivo or in vitro diffusion cell methods. This difference in percutaneous penetration is probably due to the temperature differences in the skin slab (room temperature, 20° C), in vivo (37°C) and in vitro diffusion cell (37°C) methods. It is well documented that higher temperatures results in faster rates of percutaneous penetration (23).

The in vivo and in vitro skin penetration and retention of [<sup>3</sup>H]PbTx-3 in pigs is expressed as percent dose of [<sup>3</sup>H]PbTx-3 equivalents which accumulated in each sample. These calculations were based on the amount of radioactivity in the samples and do

not take into account that a fraction of the radioactivity may have been associated with metabolites or breakdown product of [<sup>3</sup>H]PbTx-3. In the in vitro skin penetration and retention study greater than 95% of the radioactivity in each skin extract was associated with PbTx-3. These findings are consistent with the hypothesis that excised pig skin does not metabolize PbTx-3. These results are in agreement with the results of previous studies which indicate [<sup>3</sup>H]PbTx-3 is not metabolized by excised human and guinea pig skin (4).

#### CONCLUSIONS

In vitro percutaneous penetration of [<sup>3</sup>H]PbTx-3 provided an accurate indication of in vivo penetration when in vitro values were calculated by summing dermal and receptor fluid levels of [<sup>3</sup>H]PbTx-3 in the diffusion cells. The formation of a dermal reservoir of [<sup>3</sup>H]PbTx-3 in the in vivo and in vitro studies demonstrates the important role the dermis plays in the process of percutaneous absorption.

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Table 1

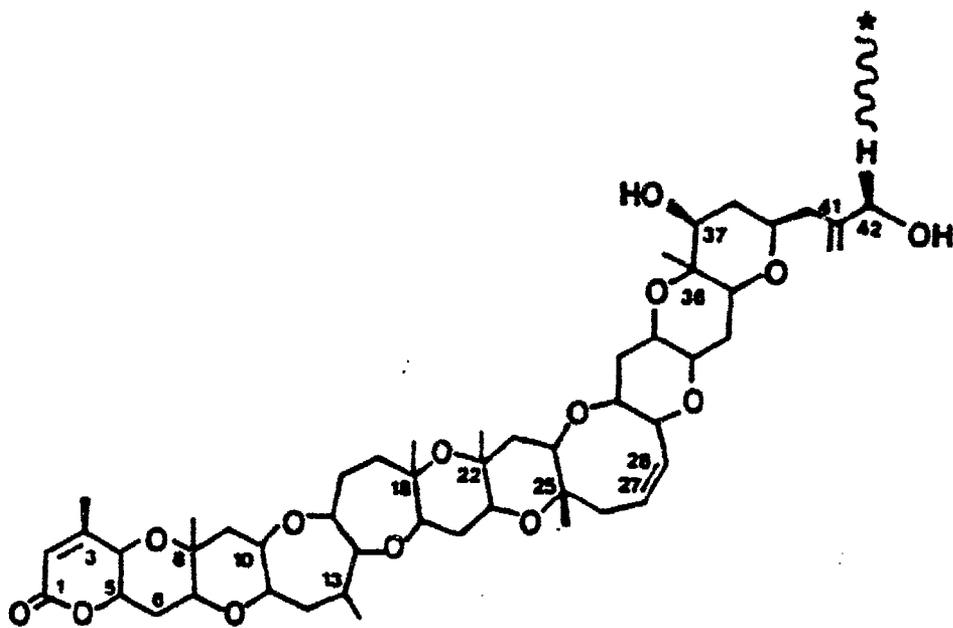
RECOVERY OF RADIOACTIVITY FOLLOWING SUBCUTANEOUS INJECTION AND  
TOPICAL APPLICATION OF [3H]PbTx-3 TO WEANLING PIGS

Organ/Tissue	Route of administration	
	Subcutaneous	Topical
Urine	11±1.8 <sup>a</sup>	1.1±0.1
Feces	8.9±7.4	1.2±0.3
Muscle	12±1.5	---
Liver	3.6±0.2	---
Kidney	0.07±0.06	---
Spleen	0.12±0.01	---
Skin surface <sup>c</sup>	--- <sup>b</sup>	34±4.8
Epidermis <sup>c</sup>	---	1.7±0.3
Dermis <sup>c</sup>	---	1.8±0.8
Subcutaneous fat <sup>c</sup>	---	0
Protective patch	---	39±20
Total recovery of dose	36±21	80±16

<sup>a</sup>Figures are expressed as percentage of dose (mean ± standard error, sample size = 3).

<sup>b</sup>---Indicates value not determined.

<sup>c</sup>At site of topical application.



Brevetoxin

[42-<sup>3</sup>H]PbTx-3

Figure 1. Structure of tritium labeled PbTx-3.

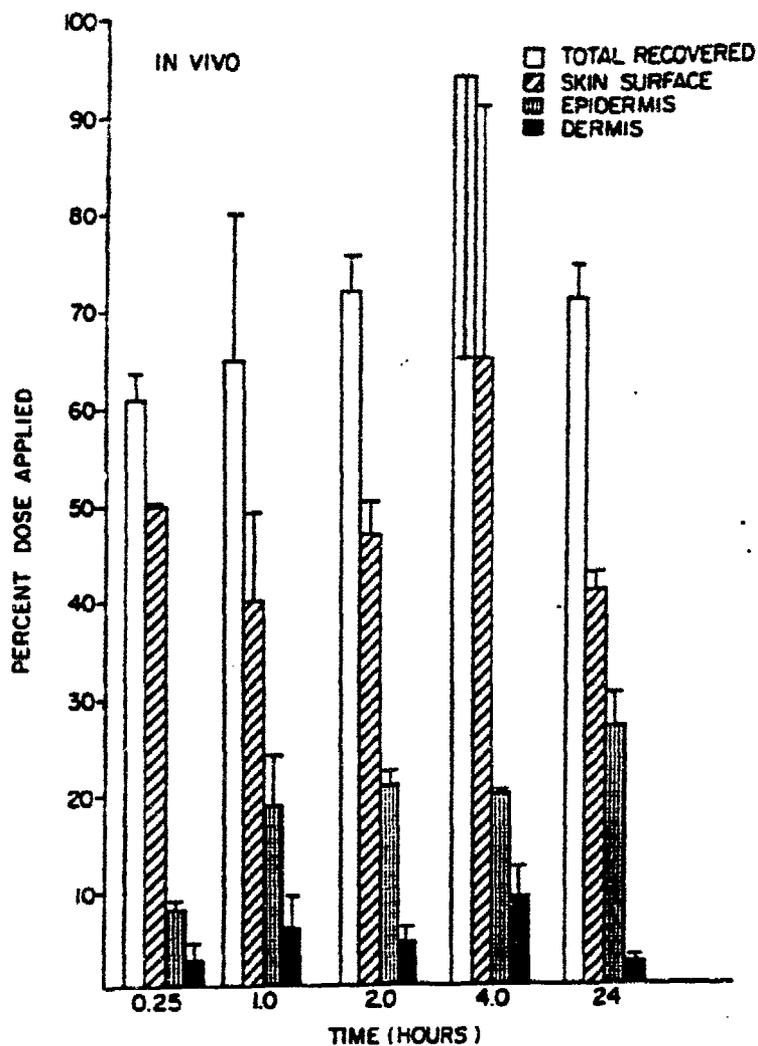


Figure 2. In vivo skin retention of PbTx-3 equivalents (percent of applied dose) following topical application to pigs. The bars represent mean recovery in the skin section and the variability is expressed as standard error of the mean.

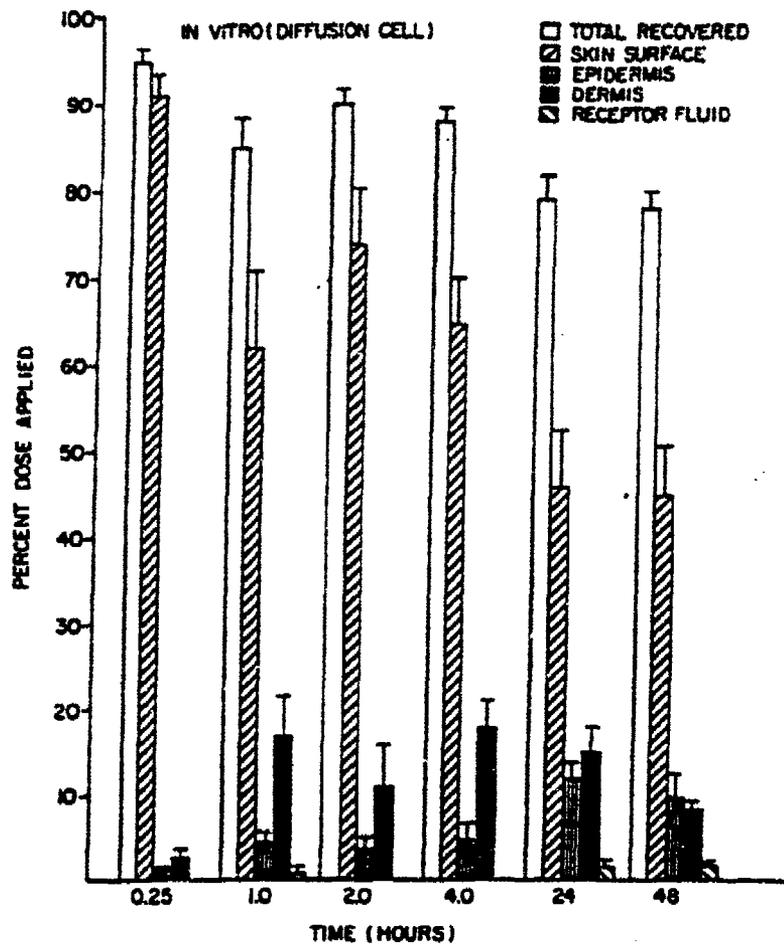


Figure 3. In vitro skin penetration and retention of PbTx-3 equivalents (percent of applied dose) following topical application to pig skin mounted on diffusion cells.

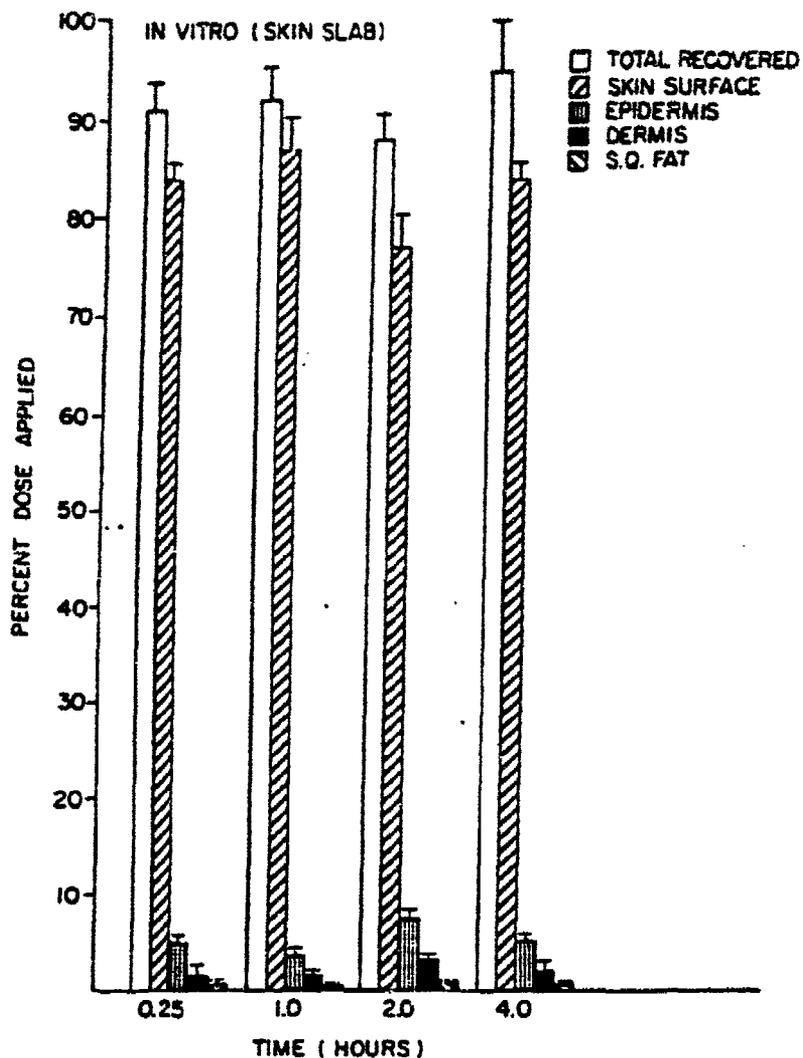


Figure 4. In vitro skin penetration and retention of PbTx-3 equivalents (percent of applied dose) following topical application to pig skin slabs incubated on absorbent material moistened with tissue culture media.

EFFECT OF VEHICLE (METHANOL, DIMETHYLSULPHOXIDE [DMSO] AND WATER) ON THE PENETRATION AND DISTRIBUTION OF BREVETOXIN (PbTx-3) INTO GUINEA PIG SKIN IN VITRO.

STATEMENT OF THE PROBLEM

In vitro methods have been widely used to predict in vivo penetration of compounds through skin. Many of these studies did not take into account the accumulation of penetrants in the layers of the skin (i.e., stratum corneum, epidermis and dermis), but instead skin penetration calculations were based only on the amount of penetrant recovered in the receptor fluid (1, 2). Recent studies indicate that in vitro skin penetration correlates better with in vivo skin penetration when in vitro values are calculated by summing penetrant recovered from the dermis and receptor fluid (3,4). Therefore, the specific objective of this experiment was to use a flowing diffusion cell method to compare the penetration and distribution of brevetoxin (PbTx-3) into receptor fluid and the layers (stratum corneum, epidermis and dermis) of excised guinea pig skin. Methanol, dimethylsulfoxide (DMSO) and water were used as the vehicles.

BACKGROUND

In preliminary in vitro experiments the penetration of PbTx-3 through skin was determined by measuring accumulation of penetrant in receptor fluid (5). Calculations of in vitro skin penetration based only on the amount of the applied dose recovered from the receptor fluid significantly under estimated skin penetration by lipophilic compounds (3, 4). PbTx-3 is a lipophilic compound which has a solubility in water of approximately 100 mg/l (Baden, personal communication). Therefore it was important to determine the effect of vehicle on the penetration and distribution of brevetoxin into the layers of the skin and receptor fluid.

## RATIONALE USED IN CURRENT STUDY

An in vitro method has been developed using glass flow-through diffusion cells for measuring the penetration of compounds through excised skin. The validity of using excised skin to estimate in vivo skin penetration by xenobiotics was tested by comparing the in vitro and in vivo skin penetration of several compounds (1, 6). It was concluded that there was good correlation between in vitro and in vivo skin penetration for most cases studied. However, there were some exceptions. The decreased correlation was attributed to low solubility of lipophilic compounds in the aqueous receptor fluid. Better agreement between in vitro and in vivo penetration through pig skin was obtained by summing the penetrant recovered from both the dermis and the penetration cell fluid to provide an adjusted in vitro penetration value (3). Therefore, the effect of vehicle on percutaneous penetration of PbTx-3 was determined by measuring accumulation of penetrant in the dermis and receptor fluid.

## MATERIALS AND METHODS

Hartley guinea pigs used in the study were obtained from the Auburn University laboratory animal facility. Male guinea pigs were chosen, ranging from 709-1,081 grams dead weight. The labelled compound ( $[^3\text{H}]\text{PbTx-3}$ ) had a specific activity of 9.93 and 13.23 Ci/m mole. The radiochemical purity was 97% or greater, as determined by high performance liquid chromatography. Prior to each experiment a guinea pig was killed with  $\text{CO}_2$ , the dead weight recorded, the hairs on the abdominal area were carefully clipped with an electric clipper and the skin removed with the aid of a scalpel blade and handle. Remaining fat or muscle was removed by blunt dissection. Disks of skin (surface area =  $1 \text{ cm}^2$ ) were prepared from the skin sample with a cork borer and scissors. The skin disks were mounted on flow-through diffusion

cells designed for the study of percutaneous penetration (Laboratory Glass Apparatus, Berkely, CA; Figure 1). The epidermal surfaces were exposed to room air and the dermal surfaces were bathed by flowing receptor fluid. The receptor fluid (Hanks's Balanced Salt Solution [HBSS] with gentamicin) was pumped through the cells at a rate of 2.9 - 3.2 ml/hour by a peristaltic pump from a reservoir. The reservoir was continuously supplied with a slow stream of gaseous mixture of 95% O<sub>2</sub>: 5% CO<sub>2</sub>. A fraction collector was used to collect hourly samples of the receptor fluid, which were then transferred to glass scintillation vials for radioactive determination. When a steady rate of receptor fluid flow was reached, the epidermal surfaces were dosed with 320 ng/cm<sup>2</sup> of [<sup>3</sup>H]PbTx-3 dissolved in 50 µl of the appropriate vehicle (methanol, DMSO or water). The diffusion cells were incubated for various time periods (0.25 to 48 hr), after which the cells were disassembled. The receptor fluid from each cell was individually transferred to glass scintillation vials for determination of radioactivity by liquid scintillation (LSC) counting. At the end of each specified time period the epidermal surfaces were washed first with cotton swabs moistened with a dilute soap solution (1:100) and then with distilled water. The cotton swabs were extracted with 10 ml of methanol. The skin disks were removed, mounted on plastic embedding blocks, and stored in the freezer (-20°C) until the following day when they were sectioned into the skin layers (epidermis, dermis) with a microtome (Bausch and Lomb Optical Co., Rochester, NY). Each skin layer was individually extracted with 2 ml of methanol. Duplicate samples (50 µl) of each extract of the surface washes and skin layers were placed in glass scintillation vials and prepared for radioactive counting by addition 10 ml of scintillation cocktail. The amount of radioactivity in the receptor fluid, surface washes and skin layers were determined with a Packard liquid scintillation counter (Model 1500, Downers

Grove II). Recovery of the applied dose (expressed as percent) was calculated by summing radioactivity in the surface wash, skin layers and receptor and fluid.

The results were statistically analyzed with SAS (Barr et al., 1979). The raw data (percent of applied dose) for each component of the diffusion chamber (surface wash, stratum corneum and epidermis, dermis and receptor fluid) and each vehicle (water, methanol, and DMSO) were fitted to three regression models (linear, quadratic, cubic). The best fit was judged by a combination of visually comparing the raw data to the predicted values generated by the analysis, the magnitude of the correlation coefficient ( $R^2$ ) and the size of the t statistic for each parameter of the model.

## RESULTS AND DISCUSSION

### Recovery for the Surface Wash

The results for each time period (0.25 to 48 hrs) and each vehicle (methanol, dimethylsulfoxide and water) are presented in Figure 2. The predicted values and 95% confidence limits<sup>ARE SHOWN</sup>.

Recovery from the skin surface was relatively constant during the first 2 hrs, but there was a consistent decline during the subsequent time periods. This was more obvious for water and DMSO than for methanol, and corresponded with increasing amounts of the applied dose penetrating into the skin layers. The penetration of PbTx-3 into and through the skin is related to the volatility and nature of the vehicles. Scheuplein et al., (7) reported that the stratum corneum is most effective as a barrier when dry, less effective when solvated with DMSO. Methanol rapidly evaporates from the skin surface due to its high volatility, and therefore appears to have minimal effects on penetration enhancement.

### Recovery From the Stratum Corneum Plus Epidermis

Recovery of radioactivity from the stratum corneum plus epidermis at 24 hr was the highest when DMSO was the vehicle, followed by water, and least for methanol (Figure 3). This finding is in agreement with Franz, (8) who found that DMSO enhanced the penetration of hydrocortisone and other compounds into the skin. The amount recovered in the stratum corneum and epidermis was relatively constant after 4 hrs.

Recovery of PbTx-3 from the stratum corneum plus epidermis 48 hr time periods was greatest when water was the vehicle, followed by DMSO and least for methanol. However, in the earlier time periods (0.25 to 2 hrs) methanol and DMSO showed higher values than water for amounts penetrating into the stratum corneum plus epidermis. This observation may be due to too short a time period (0.25 to 2 hr) for water to adequately hydrate that stratum corneum. During longer periods of exposure (4 to 48 hr) there was enough time for greater hydration. It is known that partial hydration of the stratum corneum increased permeability in vitro (6). Therefore, more complete hydration would be expected to have a more profound effect, as supported by our observations.

The percentage of the dose entering the stratum corneum plus epidermis was less than the values for the dermis for DMSO.

### Recovery from the Dermis Receptor Plus Fluid

Recovery from the dermis plus receptor fluid was minimal for methanol for all time periods (Figure 4). However, when DMSO was the vehicle recovery remained fairly low for the first 4 hr but then increased to mean values to 25% and 12% after 24 and 48 hr (respectively). This finding may be related to the solubilizing property of DMSO.

### Total Recovery from the Applied Dose

The total amount of dose recovered at the end of each experiment ranged from 72 to 94% of the dose (Figure 5). The recovery was fairly consistent throughout all the time periods for both methanol and water. When DMSO was the vehicle the values were somewhat lower for the 24 and 48 hr time periods. These time periods also corresponded to low percentage recovery from the surface wash and much higher percentages penetrating into the skin layers and receptor fluid. The reasons for these lower recovery values are unknown but may be due to less efficient extraction of [<sup>3</sup>H]PbTx-3 from skin layers than from skin surface.

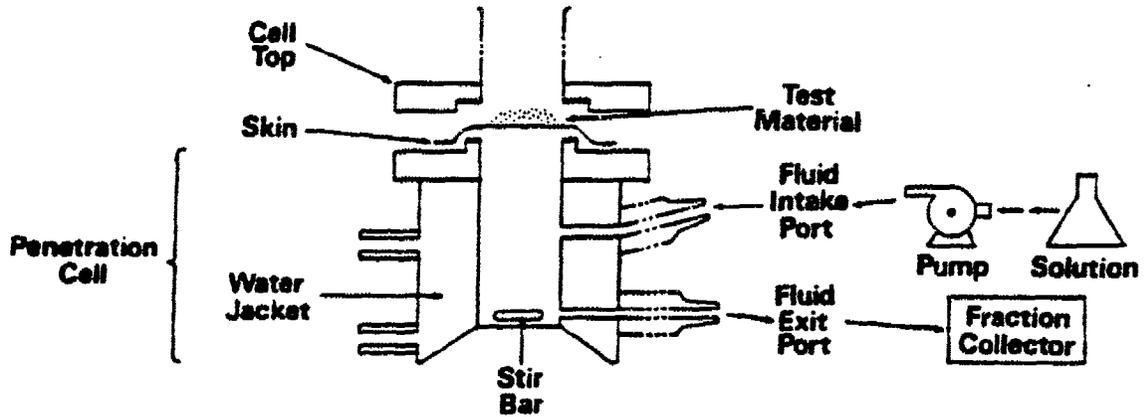
### CONCLUSION

The total amount of the dose which penetrated into the dermis and receptor fluid at 24 hrs when DMSO was the vehicle (25%) was five times that which occurred when methanol was the vehicle (5%). The percentage of the dose recovered from the receptor fluid was minimal for the vehicles methanol and water and this may be due to limited solubility of the lipophilic PbTx-3 in the aqueous receptor fluid. These results indicate that PbTx-3 readily penetrates into the dermis and receptor fluid in the presence of DMSO.

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## IN VITRO FLOW-THROUGH DIFFUSION CELL



Scale: 1 cm

Figure 1. Diagram of glass flow-through diffusion chambers used for in vitro percutaneous penetration studies.

## Surface Wash

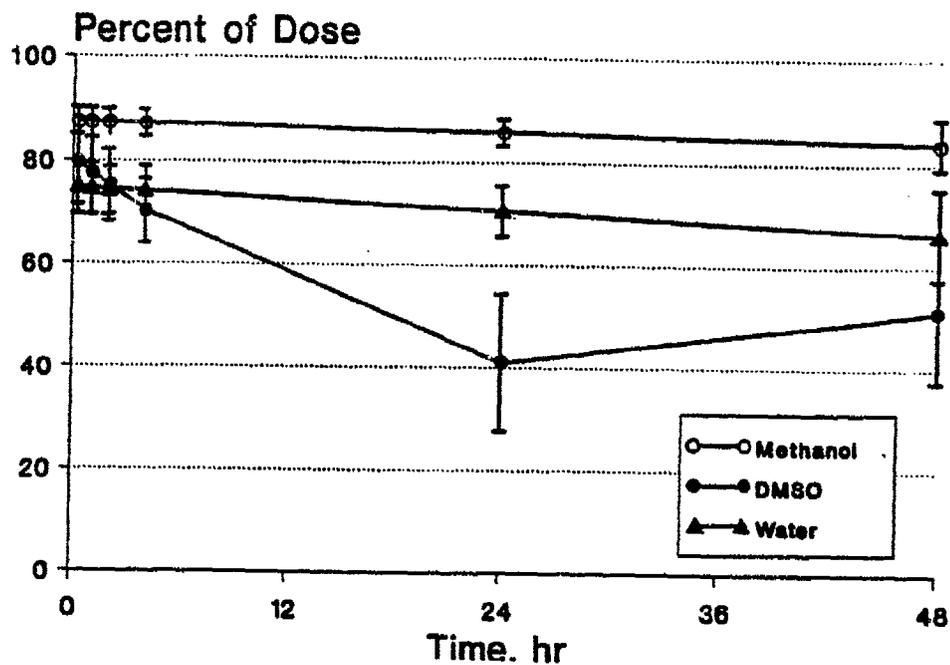


Figure 2. Effect of vehicles on the recovery of radioactivity from the skin surface of excised guinea pig skin dosed with  $[^3\text{H}]\text{PbTx-3}$ . Results are expressed as the predicted values generated by a regression analysis of the data. Variability is expressed as the 95% confidence interval.

## Stratum Corneum Plus Epidermis

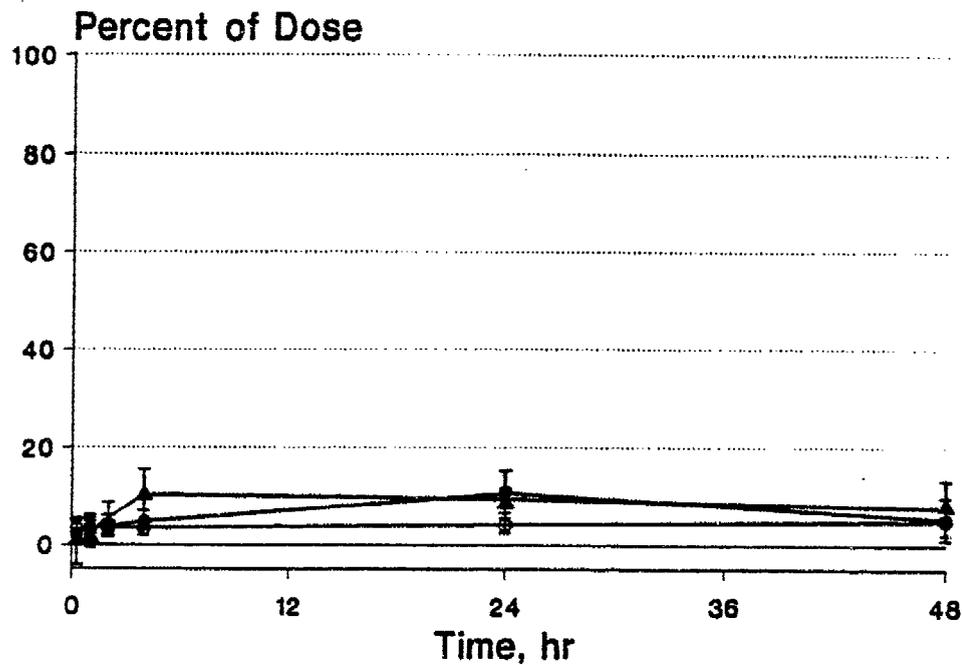


Figure 3. Effect of vehicles on the recovery of radioactivity from the stratum corneum plus epidermis of excised guinea pig skin dosed with [ $^3\text{H}$ ]PbTx-3. See Figure 2 for key to vehicles and description of values and variability.

## Dermis Plus Receptor Fluid

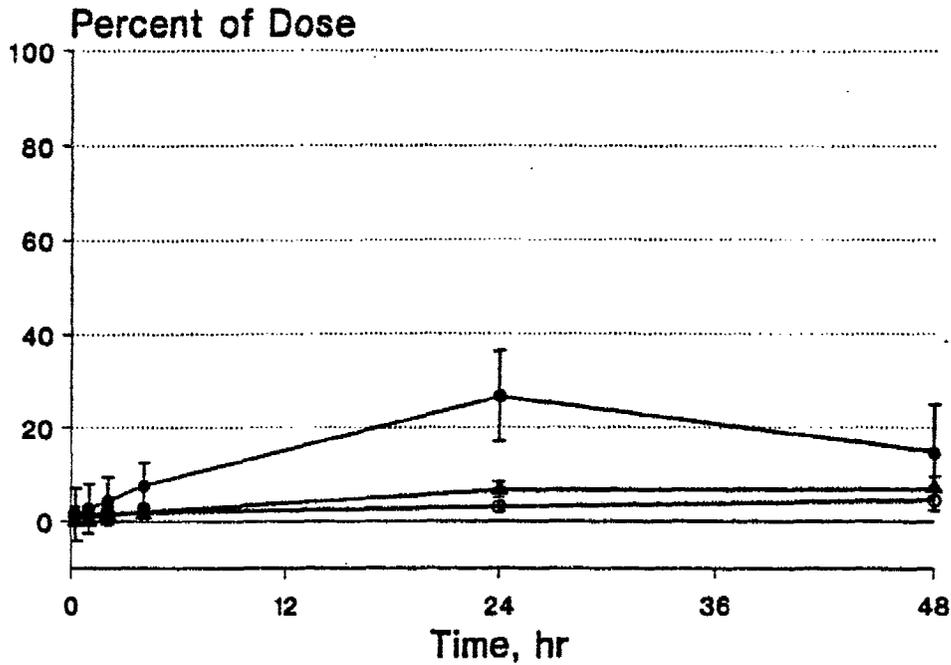


Figure 4. Effects of vehicles on the recovery of radioactivity from the dermis plus receptor fluid of excised guinea pig skin dosed with [ $^3\text{H}$ ]PbTx-3. See Figure 2 for key to vehicles and description of values and variability.

## Total Recovery

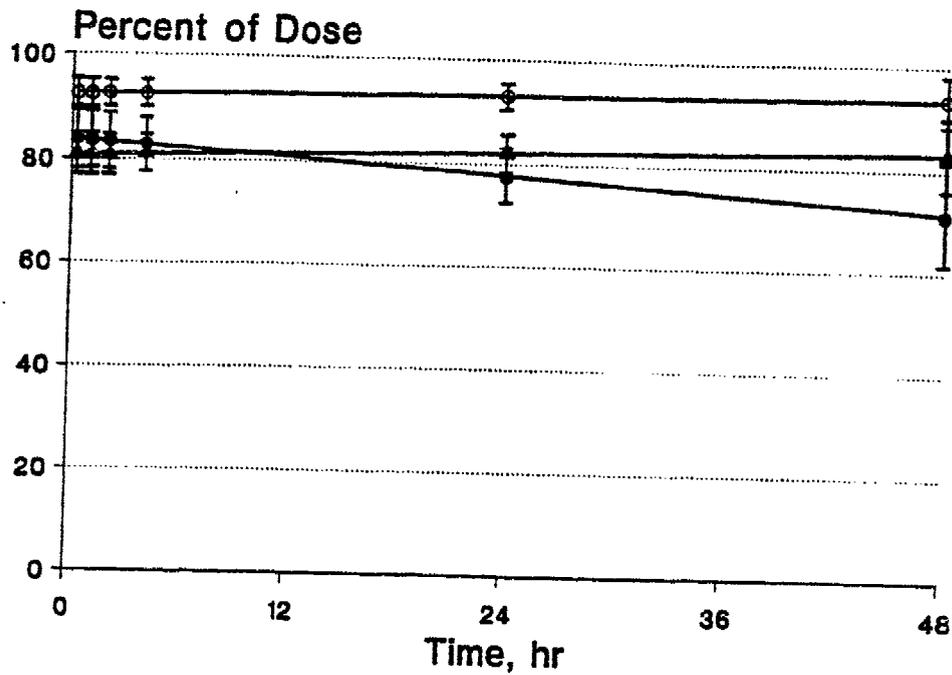


Figure 5. Effects of vehicles on the total recovery of radioactivity from excised guinea pig skin dosed with [ $^3\text{H}$ ]PbTx-3. See Figure 2 for key to vehicles and description of values and variability.

In Vitro penetration of tritium labelled water (THO) and [<sup>3</sup>H]PbTx-3 (a red tide toxin) through monkey buccal mucosa and skin.

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#### STATEMENT OF PROBLEM

The primary purpose of this work was to determine the penetration of PbTx-3 through monkey buccal mucosa and skin.

#### BACKGROUND

PbTx-3 is one of a group of brevetoxins produced by a marine dinoflagellate Ptychodiscus brevis. Blooms of this dinoflagellate are referred to as red tide. This algae has a soft outer covering which ruptures in the surf and results in the release of toxins. Blooms of P. brevis are responsible for massive fish kills in the Gulf of Mexico and along the Florida coast [1]. The red tides are also implicated in human intoxication resulting from ingestion of contaminated shellfish or inhalation of sea-spray aerosols. Persons swimming in 'red tides' may experience eye and skin irritation and itching. Ingestion of shellfish contaminated with brevetoxins causes nausea, cramps, paresthesia of the lips, face and extremities, occasionally weakness and difficulty in movement, even apparent paralysis, seizures and

coma [2].

The lipid soluble toxins produced by P. brevis are of two types, one of which is hemolytic and the other neurotoxic [3]. PbTx-3 is a neurotoxin which has a unique structure of a cyclic polyether with a molecular weight of 898 [4]. The in vivo experimental effects of P. brevis are both gastrointestinal and neurological in nature [5]. The LD<sub>50</sub> of PbTx-3 for female mice was determined to be 4 ug/kg, i.p. [6].

#### RATIONALE USED IN CURRENT STUDY

Due to the extremely toxic nature of PbTx-3, these studies can not be conducted in human beings. Comparative studies have indicated that skin absorption in monkeys approximates skin absorption in humans [7,8,9]. Accordingly, buccal mucosa and skin from rhesus monkeys was used to assess the penetration of tritiated water (THO) and PbTx-3. In addition, the penetration of THO through human skin was determined so that we could compare our results to published values for humans. To our knowledge, no single report has appeared describing the penetration of brevetoxin through buccal mucosa. The degree of correlation between in vivo and in vitro skin penetration depends on the chemical properties of the penetrant and methodology. In general in vivo skin penetration has been found to correlate well with in vitro skin penetration [10,11,12].

## MATERIALS AND METHODS

THO was generously provided by Dept. of Radiological Safety, Auburn University, AL. [ $^3\text{H}$ ]PbTx-3 was purchased from Dr. D.G. Baden (University of Miami, FL). The specific activity was 12.3-14.3 Ci/mM. The radiochemical purity of [ $^3\text{H}$ ]PbTx-3 was determined with reverse phase high pressure liquid chromatography (HPLC) prior to each experiment. HPLC was performed with a unit from Waters Associates (Milford, MA) equipped with a variable wavelength detector. For radiochromatographic analysis of [ $^3\text{H}$ ]PbTx-3, a radioactive flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL) was attached to HPLC system. The scintillation cocktail was Flo-scint (Radiomatic). The HPLC column was a 10  $\mu\text{m}$  C<sub>18</sub> uBondapak steel column (2 mm x 36 cm). The eluant was methanol:water (85:15) pumped at a rate of 0.5 ml/min and ultraviolet (UV) absorbance was monitored at 215 nm. The radiochemical purity of [ $^3\text{H}$ ]PbTx-3 was determined to be more than 99 percent. Retention times of [ $^3\text{H}$ ]PbTx-3 and contaminant peak were 4.0 to 4.2 min and 1.0 to 1.3 min, respectively.

Rhesus monkey buccal mucosa and skin were obtained from Wisconsin Regional Primate Research Center (Madison, WI), Delta Primate Center (Covington, LA), California Regional Primate Research Center (Davis, CA) and Washington Regional Primate Research Center (Seattle, WA). The monkeys were sacrificed as a part of other ongoing studies. Collected tissues were packaged with wet ice and shipped by overnight delivery. Tissues were used immediately on arrival. Human cadaver abdominal skin was obtained from local hospitals and was stored at 4°C for up to 5

days prior to use. Previous studies [13] have shown that the permeability of skin did not change significantly when stored by this method for up to 10 days. Before each experiment, the outer surface of the tissue disks was carefully examined to determine if the epithelial or epidermal surface was intact. The tissue disks were discarded if the surface was damaged. It is widely accepted that xenobiotics penetrate through skin by passive diffusion [14]. The main "barrier" to penetration is the stratum corneum [14]. The metabolic capacity of skin has been shown to have minimal effect on the rate of percutaneous penetration of xenobiotics [15].

Outer surface of the monkey skin was carefully shaved with electric clippers (Oster Model A-2, Milwaukee, WI). Loose fat and connective tissue was removed from the inner surface (dermal side) of buccal mucosa and skin. Excised tissue discs had a diffusible surface area of  $2.8 \text{ cm}^2$  and were mounted on static teflon diffusion cells, Fig. 1, [16]. The diffusion cells have a head pressure reservoir which served to (i) equalize the pressure between the receptor chamber and the environment and (ii) as a port for the replacement of receptor fluid which was removed at sampling periods. The donor (epidermal surface) and receptor chambers (dermal surface) of diffusion cells were filled with Hanks' Balanced Salt Solution (HBSS) with HEPES buffer (10 mM liter) and gentamicin (50 mg/liter). The pH of HBSS was adjusted to 7.3. Several groups of assembled diffusion cells were incubated at  $4^\circ\text{C}$  for 15 hr in order to fully hydrate the tissue discs

prior to penetration experiments. The receptor fluid was bubbled with O<sub>2</sub>:CO<sub>2</sub> (95:5) to maintain the viability of the tissue discs [17]. One group of the buccal mucosal discs was placed in between two layers of large pore nylon membranes (Cole Parmer, Chicago, IL) in order to determine if it was necessary to use additional support for the delicate buccal mucosa. One group of monkey skin discs was also placed in between the nylon membranes to determine if there was hindrance to penetration due to the nylon membranes. At zero time, the HBSS on outer surfaces was replaced with dose. The dose, expressed as total amount of material applied, was 0.8-1.0 uCi of THO diluted in 2 ml of water or 371-412 ng of [<sup>3</sup>H]PbTx-3 (5-7 uCi) dissolved in 2 ml of water. The epidermal surfaces were occluded for the entire exposure period (12-24 hr). The diffusion cells were incubated in an environmental chamber at 37°C in order to maintain them at a constant temperature. Sequential samples (50 ul) were collected from the dermal side (receptor fluid) every 15 min during the first hr, every 30 min from 1 to 4 hr and every one hr until 24 hr. Fresh HBSS was added to the receptor chambers after each sample in order to replace the amount that was removed. At the end of each experiment, the dose left on the epidermal surface was collected and measured. Epidermal surfaces were gently washed with cotton swabs moistened with soap and water (1:10 dilution) in order to determine the amount of dose remaining on the epidermal surface. Cotton swabs and tissue discs from the diffusion cells dosed with THO or [<sup>3</sup>H]PbTx-3 were placed in vials

containing 10 ml of water or methanol, respectively. Extraction of the radiolabeled compounds from the tissue was facilitated by sonicating the vials for 10 minutes. Radioactivity in the receptor fluid, skin wash, skin extracts and dose left on the epidermal surface was assessed by mixing aliquots of each sample with scintillation cocktail (Hydrofluor, National Diagnostics, Manville, New Jersey) and then measuring the radioactivity in each vial with a Liquid Scintillation Counter (Packard Instrument, Downersgrove, IL).

The distribution of [ $^3\text{H}$ ]PbTx-3 into the layers of buccal mucosa (epithelium and lamina propria) or skin epidermis and dermis) was determined. Tissue discs were immediately frozen ( $-20^\circ\text{C}$ ) at the end of the exposure period and sectioned with a microtome (Bauch & Lomb, Optical Co., Rochester N.Y.) at a setting of 25  $\mu\text{m}$ . Outer 100  $\mu\text{m}$  was sectioned as epidermis/epithelium. Dermis was 450-550  $\mu\text{m}$  and lamina propria was 1000-1100  $\mu\text{m}$ . Tissue slices were extracted in methanol and radioactivity was determined with standard liquid scintillation methods.

The permeability coefficient ( $K_p$ ) for THO was calculated by dividing the net total transfer of compound during each time interval by the amount of the compound in the donor side solution and the surface area of the tissue disc available for diffusion. For each treatment group,  $K_p$  (mean  $\pm$  SD) was calculated from all time intervals.

$K_p$  is related to the total net flux by the equation:

$$K_p = J / \Delta C \times A$$

where  $J$  = net flux of the compound (ng/hr), which is the amount penetrated into the inner chamber from the outer chamber, in a given period of the time;  $\Delta C$  = the concentration difference (ng/cm<sup>3</sup>) across the tissue disc;  $A$  = the surface area (cm<sup>2</sup>) of the tissue disc exposed to the compound, [18].

The stability of [<sup>3</sup>H]PbTx-3 in the receptor fluid (HBSS) was evaluated by mounting Teflon discs, instead of tissue discs, on one or two diffusion cells in each experiment. The receptor fluid was dosed with a fraction of the dose of [<sup>3</sup>H]PbTx-3 prepared for the respective experiment. The diffusion cells containing Teflon discs were incubated at 37°C along with the diffusion cells containing tissue discs. Receptor fluid samples from the diffusion cells containing teflon or tissue discs were treated in the same manner. The stability of [<sup>3</sup>H]PbTx-3 under experimental conditions was determined. At the end of each experiment, tissue discs dosed with [<sup>3</sup>H]PbTx-3 were extracted with methanol and radiochemical analysis was done with HPLC. Similarly, aliquots from receptor fluid were also analyzed.

Statistical analyses of data was performed by comparing means using Student's t test. The level of significance was set at  $P < 0.05$ .

## RESULTS AND DISCUSSION

The permeability coefficients for buccal mucosa and skin are listed in Table 1. The buccal mucosa was generally in the range of 1000 - 1200  $\mu\text{m}$  thick. The thickness of the monkey skin and human skin was in the range of 700 - 800  $\mu\text{m}$  thick. In order to facilitate comparison, the  $K_p$  of skin and buccal mucosa have been adjusted to a thickness of 750  $\mu\text{m}$  thick, as described by Galey et al., [18]. The permeability of excised human skin was determined to correlate favorably with other reports [19]. Hydration of human skin (prior to penetration experiment) almost doubled the permeability of human skin to THO. Hydration has the effect of swelling the cells and reducing the density of their structure and their resistance to diffusion. Hydration of the stratum corneum can increase diffusion constants up to 10 fold [18]. No significant difference ( $P > 0.05$ ) was observed between permeability of hydrated human skin and hydrated monkey skin. Percutaneous absorption in rhesus monkey is generally similar to that in man [21,22,23].

Monkey buccal mucosa was determined to be eight times more permeable to THO than monkey skin. The larger  $K_p$  of monkey buccal mucosa than monkey skin for THO is in agreement with the generally held view that buccal mucosa is more permeable than skin. The greater permeability of monkey buccal mucosa is probably due to the lack of keratinized epithelium [24]. Nylon membranes did not alter the penetration of THO through monkey buccal mucosa or skin ( $P > 0.05$ ). It was also concluded that it

is not necessary to provide additional support to the delicate buccal mucosa.

Preliminary analysis of the accumulation of [ $^3\text{H}$ ]PbTx-3 in receptor fluid bathing the inner surfaces of monkey buccal mucosa and skin indicated a long lag phase and slow penetration rate (Fig. 2). These findings are consistent with other reports where diffusion of lipophilic penetrants into receptor fluid is minimal due to limited solubility in the aqueous fluid [25]. Wannemacher et al., [26] have reported that PbTx-2 (a related compound) has poor solubility in water (< 100 ug/L). This has been further confirmed by Dr. D. Baden (personal communication) who estimates the solubility of PbTx-3 in water to be 100 ug/L. Therefore, instead of determining the  $K_p$  of buccal mucosa and skin to [ $^3\text{H}$ ]PbTx-3, the distribution of radioactivity in monkey buccal mucosa and skin following topical application of [ $^3\text{H}$ ]PbTx-3 was determined (Table 2). At each time interval, the largest portion of the applied dose was recovered from the tissue surface (epithelial or epidermal). As early as 15 min after dosing, [ $^3\text{H}$ ]PbTx-3 had penetrated into the epithelium/epidermis and lamina propria/dermis. No uniform pattern was observed for penetration into the epithelium/epidermis. The percent of the dose that penetrated into the lamina propria/dermis increased with time (15 min to 24 hr). After 24 hr, 34 and 13 percent of the dose had penetrated into the lamina propria and dermis, respectively. [ $^3\text{H}$ ]PbTx-3 recovered from receptor fluid was less than 2 percent for all the time intervals studied (e.g., 15 min to 24 hr).

In contrast to the large amount of PbTx-3 recovered from the lamina propria/dermis, after 24 hr, only 2 and 8 percent of THO was residing within the buccal mucosa and skin, respectively. It has been reported that during in vitro studies the dermis acts as an artificial reservoir for lipophilic compounds but not for hydrophilic compounds [12]. Hawkins et al., [12] and Kemppainen et al., [27] have determined that in vitro skin penetration of lipophilic compounds correlates better with in vivo skin penetration when in vitro values are calculated by summing the amount of penetrant in dermis and receptor fluid. In all the experiments, total recovery of THO was 100-101%.

The calculations of diffusion of PbTx-3 in buccal mucosa and skin were based on quantification of radioactivity in each sample. Radiochemical analysis of the samples was done to determine if the radiolabeled PbTx-3 was stable under experimental conditions. The stability of [<sup>3</sup>H]PbTx-3 in receptor fluid (HBSS) was determined at 37°C. The [<sup>3</sup>H]PbTx-3 preparation breaks down at the rate of approximately 10% during 24 hours. Radiochromatographic analysis of samples from the skin surface, skin extract and receptor fluid indicated that between 85 and 95% of the radioactivity was associated with [<sup>3</sup>H]PbTx-3. The remaining radioactivity (5 to 15%) in each sample was associated with a breakdown product which had a retention time of 1 to 1.3 min. These results indicate that at least 85% of the radioactivity in each sample was associated with PbTx-3.

In conclusion, PbTx-3 readily penetrates into the layers of buccal mucosa/skin, but probably, due to its low solubility does not diffuse into receptor fluid. Therefore, when using an in vitro methods to assess the penetration of lipophilic compounds through tissue it is important to determine the distribution into layers of buccal mucosa and skin.

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Table 1. Permeability Coefficient of buccal mucosa and skin<sup>a</sup> to tritiated water (THO)

Tissue	Kp cm/hr, mean $\pm$ SD $\times 10^{-3}$
Hydrated MS (11)[6] <sup>b</sup>	0.77 $\pm$ 0.57
Hydrated MSN (7)[3]	0.65 $\pm$ 0.24
Hydrated Monkey BM (8)[3]	6.15 $\pm$ 2.57
Hydrated Monkey BMN (4)[3]	5.90 $\pm$ 1.94
Human Skin (not hydrated) <sup>c</sup> (7)[3]	0.47 $\pm$ 0.15
Hydrated Human Skin (7)[3]	0.88 $\pm$ 0.42

<sup>a</sup> MS = Monkey skin, MSN = Monkey skin supported by nylon, BM = Buccal Mucosa, BMN = Buccal mucosa with nylon.

<sup>b</sup> ( ) total number of replicates, [ ] number of donor individuals.

<sup>c</sup> Hydrated or not hydrated refers to whether or not tissues were incubated (4°C) in Hanks Balanced Salt Solution prior to penetration study.

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Table 2. Distribution of [<sup>3</sup>H] PbTx-3 in monkey buccal mucosa and skin after topical application

Time After Dosing	Barrier	(n) [N] <sup>a</sup>	Percent of dose (Mean ± SD) recovered				Total Recovery
			Skin Surface <sup>b</sup>	Epithelium <sup>c</sup> / Epidermis	Lamina propria/ Dermis	Receptor Fluid	
15 Min	BM <sup>e</sup> MS	(3) [3] (3) [3]	87 ± 4.4	0.34 ± 0.12	1.3 ± 0.13	0.02 ± 0.01	95 ± 4.5 92 ± 6
			83 ± 3.6	1.1 ± 0.11	1.5 ± 0.14	0.006 ± 0.004	
2 hr	BM MS	(3) [3] (3) [3]	91 ± 7	0.62 ± 0.05	3.5 ± 0.77	0.02 ± 0.01	95 ± 5.6 97 ± 7.6
			95 ± 5	0.74 ± 0.46	1.5 ± 0.60	0.01 ± 0.01	
4 hr	BM MS	(3) [3] (3) [3]	84 ± 6.4	1.4 ± 0.70	6.4 ± 1.7	0.43 ± 0.31	92 ± 7 95 ± 8.5
			91 ± 9	1.3 ± 0.8	3.3 ± 0.55	0.02 ± 0	
12 hr	BM MS	(3) [3] (5) [4]	71 ± 11	0.72 ± 0.37	2.2 ± 7	1.3 ± 0.62	96 ± 5.5 96 ± 6.4
			90 ± 10	1.2 ± 0.34	4.8 ± 1.9	0.10 ± 0.03	
24 hr	BM MS	(6) [4] (6) [4]	54 ± 21	2.4 ± 0.92	33 ± 9.0	1.9 ± 1.1	92 ± 3.5 93 ± 4.9
			79 ± 18	0.88 ± 0.28	12 ± 8.4	0.38 ± 0.21	

<sup>a</sup>(n) = Total number of replicates. [N] = total number of individuals.

<sup>b</sup>Includes skin wash + dose left on epithelial/epidermal surface.

<sup>c</sup>Thickness of 100 μm

<sup>d</sup>Thickness of lamina propria was 1000 - 1100 μm and of dermis was 450 to 550 μm

<sup>e</sup>BM=buccal mucosa, MS=monkey skin

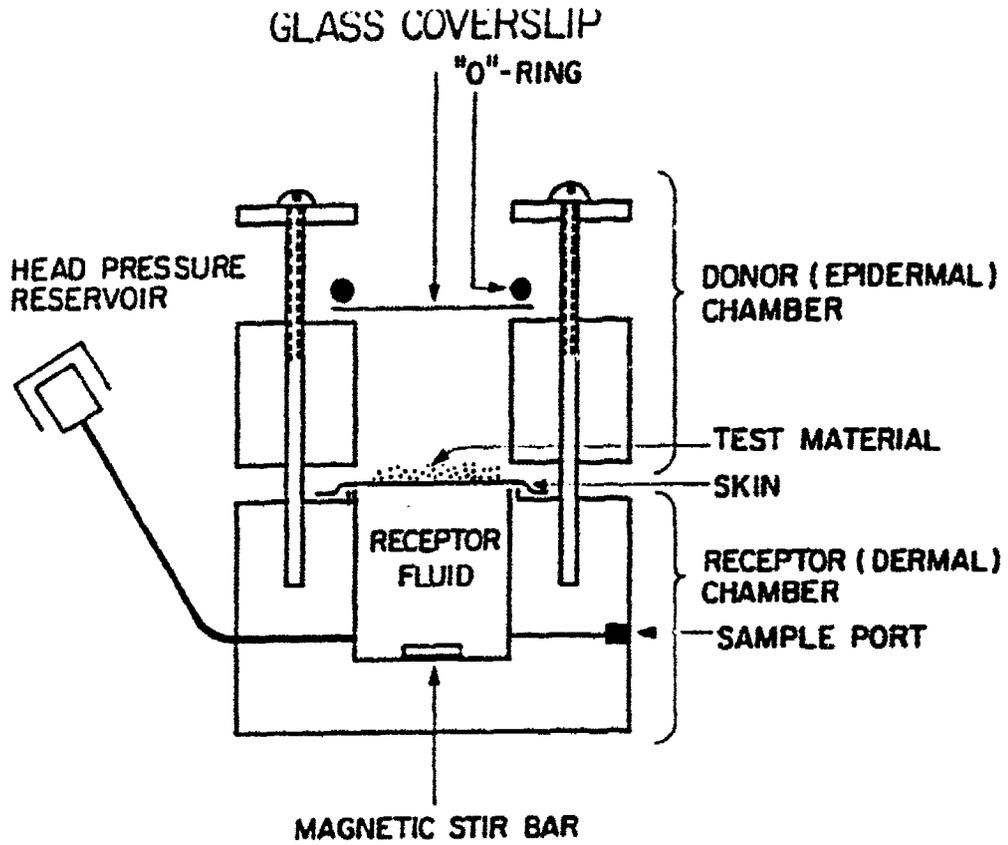


Figure 1. Teflon static diffusion cell used for buccal mucosa and skin penetration studies.

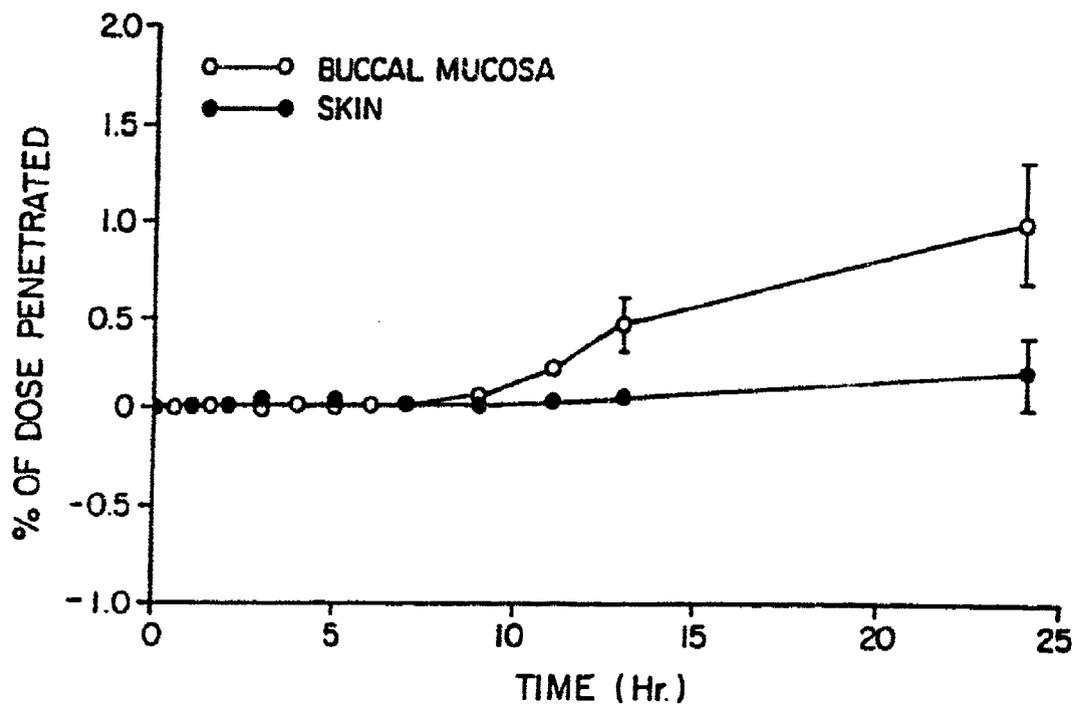


Figure 2. Accumulation of radioactivity in receptor fluid bathing inner surface of monkey buccal mucosa and skin following application of [ $^3\text{H}$ ]PbTx-3 to the outer (epidermal) surface.

IN VITRO PENETRATION AND DISTRIBUTION OF LYNGBYATOXIN A IN HUMAN  
AND GUINEA PIG SKIN

RG Stafford, BW Kemppainen, M Mehta

STATEMENT OF PROBLEM

The purpose of this study was to measure the penetration and distribution of lyngbyatoxin A in human and guinea pig skin.

BACKGROUND

Lyngbyatoxin A is a highly toxic skin irritant produced by two different organisms, a marine blue-green algae found in tropical oceans, *Lyngbya majuscula*, and a fungus-like bacterium *Streptomyces mediocidicus*. *L. majuscula* has been implicated in many occurrences of a contact dermatitis known as swimmers' itch in Hawaii and Japan. Lyngbyatoxin A has also been discovered to be a potent tumor promoter in mice and is a potential health risk because of its toxic properties (see Figure 1). The measurement of absorption of lyngbyatoxin A through skin is an important step in assessing the health risk from cutaneous exposure to lyngbyatoxin A whether it be from environmental sources or from laboratory spills when extracting and studying the compound.

RATIONALE USED IN CURRENT STUDY

In vitro methods using guinea pig and human skin were used because the extreme toxicity of lyngbyatoxin A prohibits testing using human subjects. The amount of skin penetration measured using animal models can be compared to human data. See Figure 2 of diagram of human skin.

Methods for extracting and measuring lyngbyatoxin A in biological and aqueous samples were developed. Measurement of

lyngbyatoxin A required the use of high performance liquid chromatography. Three in vitro skin penetration experiments using excised guinea pig abdominal skin and three experiments using human cadaver abdominal skin were performed. Also, the octanol/water partition coefficient was determined for lyngbyatoxin A. The partition coefficient is a measure of the solubility of a compound in two immiscible solvents (e.g. octanol and water).

#### MATERIALS AND METHODS

##### **Skin Penetration Experiments Using Lyngbyatoxin A**

1. Experimental Design: A total of six skin penetration experiments using lyngbyatoxin A were performed. Three of the experiments were performed with skin excised from three guinea pigs and three of the experiments used human abdominal skin excised from three cadavers. Three different incubation time periods were used for each experiment (1, 4 and 24 hours). One 48 hour incubation time was performed with guinea pig skin. High performance liquid chromatography (HPLC) was used to measure lyngbyatoxin A in components of skin diffusion chambers at the end of each exposure period. Methods development, recovery, extraction optimization and validations were performed. Lyngbyatoxin A used in these studies was extracted from cultures of S. mediterraneus according to the method described by Fujiki and Sugimura (1983).

2. Skin Penetration Chambers: In vitro diffusion chambers used were of the finite dose type. They were constructed of teflon in two parts (see Figure 3b) and consisted of a donor chamber and a

receptor chamber. The skin is placed horizontally between the two chambers. The donor chamber fits on the epidermal surface of the skin and the receptor chamber fits on the dermal surface. The two chambers are held vertically together by four nylon screws. The receptor chamber contains a sample port which is fitted with a silicone rubber plug (to enable sampling of receptor fluid without dismantling the chamber) and a stainless steel vent tube which allows the receptor fluid to expand and contract without putting hydrostatic pressure on the tissue. The skin is placed in the apparatus so that the dermal surface is bathed by the receptor fluid, and the epidermal surface is exposed to the donor chamber. The receptor fluid is continually stirred by a motor driven magnetic stir bar. Test material is placed on the epidermal surface of the skin and diffuses through the skin into the receptor fluid. The temperature of the diffusion chambers were maintained at 36°C by placing them in an environmental chamber. Figure 3a shows the donor chamber occluded by a glass coverslip which is tightly sealed to the upper chamber by an O-ring and screws. In this study, the skin was occluded by placing the coverslip loosely over the top of the donor chamber. The surface area of the donor chamber averaged 1.9 cm<sup>2</sup>. The volume of the receptor chamber was about 2 ml.

3. Receptor Fluid: The receptor fluid was HEPES-buffered Hanks' balanced salt solution (HHBSS, GIBCO BRL Laboratories, Life Technologies, Inc. Gaithersburg, MD) with gentamicin sulfate (50mg/liter, Sigma Chemical Co. St. Louis, MO). HHBSS is a cell culture medium demonstrated to maintain the viability of skin

during in vitro skin penetration experiments (Collier et al., 1989). HEPES is N-2-hydroxyethylpiperazine-N'-2 ethanesulfonic acid, a zwitterionic buffer with good buffering capability in a pH range of 6.7 and 8.4 (GIBCO BRL Catalogue and Reference Guide, 1990, pg 227). Gentamicin sulfate is used in receptor fluids to inhibit bacterial growth (Collier et al., 1989).

#### Recipe for HHBSS

- a. 100 ml of 10x Hanks' balanced salt solution concentrate mixed with 850 ml deionized water.
- b. Add 4.7 ml 7.5% NaCO<sub>3</sub> (w/v)
- c. Add 10 ml HEPES buffer (1 Molar)
- d. Add 1 ml Gentamicin sulfate (50 mg/ml)
- e. pH to 7.3-7.4 with HCl or NaOH
- f. Bring up volume to 1 liter using deionized water.
- g. Filter through sterile disposable filter (1 liter, 0.2  $\mu$ m CN, Nalge Company, Division of Sybron Corp. Rochester, NY)
- h. Store in refrigerator until used.

4. Preparation of Guinea Pig Skin: Three male Hartley guinea pigs (Harlan Sprague Dawley, Indianapolis, IN) weighing between 786 and 993 g were euthanized using carbon dioxide. The hair on the ventral surfaces was clipped (Oster Division, Sunbeam Corporation, Milwaukee, WI), taking care not to damage the skin. The clipped skin was removed from the animal using scissors and placed epidermal side up in a stainless steel pan on a paper towel soaked with sterile receptor fluid. The skin was covered with aluminum

foil and kept in a refrigerator (4°C) until used, not more than 24 hours later. Disks of full thickness skin approximately 2.3 cm in diameter were cut with scissors. The thickness of the skin was measured using a micrometer (Mitutoyo, Japan) in five different places on each piece.

5. Preparation of Human Skin: Shipments of human skin samples wrapped in airtight plastic bags and packed in wet ice were received from a hospital pathology department via overnight delivery. All of the skin samples were from the abdominal region of cadavers. Autopsy was performed within 24 hours of death. Upon arrival the skin was placed in the refrigerator until used, not more than 24 hours later. Before use, the skin was visually examined for intactness. Damaged, blemished or otherwise defective skin was not used. The human skin was received with a thick layer of fat on the dermal surface. A dermatome (Padgett Dermatome model B, Kansas City Assemblage Co. Kansas City, MO) was used to remove a layer of the skin (epidermis and dermis) similar in thickness to the full thickness guinea pig skin used in this study. Disks of human skin were prepared and measured in the same manner as the guinea pig skin.

6. Assembly of the Chambers: Before use, the receptor fluid (HHBSS) was aerated with 95% O<sub>2</sub>: 5% CO<sub>2</sub>. A magnetic stir bar and receptor fluid was placed in the receptor fluid chamber. A disk of skin was placed horizontally between the donor and receptor chamber. Care was taken to avoid air bubbles in the receptor

chamber. The upper and lower chambers were fastened together with screws. Receptor fluid was introduced through the vent to check the diffusion apparatus for leakage around the vent, sample port and skin. The assembled chambers were placed on a plexiglass base equipped with a magnetic stirrer and incubated in the environmental chamber for one hour prior to dosing to allow the chambers to reach thermal equilibrium.

7. Preparation of Lyngbyatoxin A: Lyngbyatoxin A was stored frozen ( $-20^{\circ}\text{C}$ ) dissolved in dimethylsulfoxide (DMSO, Aldrich HPLC grade, Aldrich Chemical Co. Milwaukee, WI) at a concentration of  $10\ \mu\text{g}/\mu\text{l}$ . The day of the experiment the lyngbyatoxin A was thawed, an aliquot withdrawn and the stock lyngbyatoxin immediately replaced in the freezer. The aliquot of lyngbyatoxin stock solution was diluted with DMSO to a concentration of  $2\ \mu\text{g}/\mu\text{l}$ . A sufficient amount of the lyngbyatoxin dilution was made up to dose the epidermal surface of each skin disc, plus a  $25\ \mu\text{l}$  excess for further dilution for use as a standard for HPLC.

8. Dosing the Chambers: Each epidermal surface was dosed with  $50\ \mu\text{g}$  of lyngbyatoxin A dissolved in  $25\ \mu\text{l}$  of DMSO on the epidermal surface with a glass microliter syringe (Hamilton Company Reno, Nevada) fitted with a blunt needle. The dose was gently distributed across the surface of the skin with the blunt tip of the needle.

9. Incubation of the Chambers: The chambers were kept in the environmental chamber at a temperature of  $36$  to  $37^{\circ}\text{C}$  for the

various time periods (1 hr, 4 hr, 24 hr and 48 hr). The receptor fluid was kept mixed throughout the experiment with the magnetic stir bars. The temperature and relative humidity were monitored during the experiment. The relative humidity was measured by means of a sling psychrometer (Taylor Instruments Division, Sybron Corp. Arden, NC) and varied from 16 to 42%, with an average relative humidity of 27%.

10. Disassembly of the Diffusion Apparatus: A small volume of the receptor fluid was withdrawn from the receptor chamber with a 1 cc tuberculin syringe (Becton Dickinson Rutherford, NJ) to prevent spilling the receptor fluid when the skin was removed. Lyngbyatoxin remaining on the surface of the skin was removed by gently washing the surface of the skin. The skin was washed gently with a ball of glass wool dampened with a dilute detergent solution (1% Liqui-nox laboratory detergent, Alconox, New York, NY), rinsed with glass wool dampened with deionized water and dried with dry glass wool. Cotton swabs are routinely used to decontaminate the skin surface, but this method was not used because of the interfering chromatographic peaks which resulted from extraction of cotton swabs. The upper section of the diffusion chamber was then removed, the skin taken off of the receptor chamber and placed epidermal side up on a mounting block (Thomas Scientific Swedesboro, NJ) with O.T.C. embedding compound (Miles Inc. Elkhart, IN). Each block containing skin was placed upside down in a petri dish on a piece of aluminum foil so that the weight of the block would keep the skin flat. The blocks were stored in a freezer (-20°C) until ready to

be sectioned on the microtome. The receptor fluid remaining in the receptor chamber was combined with the receptor fluid previously removed from the diffusion apparatus and placed in a culture tube. The receptor chamber was rinsed three times with a small volume of chloroform and added to the receptor fluid.

11. Extraction of Lyngbyatoxin A From Skin: Two experiments were performed to determine the recovery of lyngbyatoxin A from human skin. In the first experiment three pieces of human skin were injected with 1  $\mu$ g of lyngbyatoxin A. The skin discs were mounted on blocks, frozen (-20°C), sliced with the dermatome and extracted with chloroform. Recoveries averaged 65%  $\pm$  13%. Another experiment was performed with previously frozen human skin. Three discs of thawed skin were each injected with 1  $\mu$ g of lyngbyatoxin A, frozen, microtomed, extracted and quantified. Recoveries averaged 66%  $\pm$  18%.

12. Contamination of Skin Sections with Lyngbyatoxin A when Microtoming: When the discs of skin containing lyngbyatoxin A were sliced on the microtome, decontamination of the blade was performed between each layer. A check for carry over of lyngbyatoxin A between skin layers and between skin discs was performed at the same time that the recovery of lyngbyatoxin A from skin was being investigated. During the sectioning of one of the skin discs, slicing was stopped and the blade decontaminated by wiping with a glass wool ball. This was included with the skin slices. Then a glass wool ball dampened with chloroform was used to

wipe the microtome blade. This glass wool ball was checked for lyngbyatoxin A contamination. None was found. In the second decontamination experiment, a skin disc containing lyngbyatoxin A was sliced with the microtome. The mounting block was removed and the microtome blade decontaminated with glass wool moistened with chloroform. A block containing a skin disc with no lyngbyatoxin A was placed in the microtome and the skin sliced in the same manner as the previous block. The sections were extracted with chloroform. No lyngbyatoxin A was found in the skin sections.

13. Accuracy of the Microtome: The accuracy of sectioning the skin with the microtome was determined. The thickness of five pieces of guinea pig skin (average of 1050 microns) were measured, mounted and sectioned with the microtome. Sections totaling 600 microns were removed, the skin thawed and measured with the micrometer. The average thickness actually removed was 556.2 microns or 92.7% of the desired amount.

14. Histological Examination of Microtomed Skin Discs: Discs of human (n=3) and guinea pig skin (n=3) were prepared for sectioning with the microtome. One disc of each species was placed in 10% neutral buffered formalin for histological preparation. The second disc of each species was microtomed to remove the first 15 microns (stratum corneum) of the skin surface. The third disc of each species was microtomed so as to remove the first 100 microns of the skin (stratum corneum and viable epidermis). The remaining portion of the skin discs were placed in 10% neutral buffered formalin. The

preserved skin was prepared by the histology laboratory of the Pathobiology Department of Auburn University. Triplicate samples of each skin disc were embedded in paraffin, microtomed perpendicularly to the skin surface, placed on a glass slide with coverslip and stained with hematoxylin and eosin stain.

The prepared slides were viewed under a microscope using 100X magnification. The skin on all slides was wrinkled but the stratum corneum, epidermis and dermis could easily be seen and the layers could be distinguished from each other. All the skin layers were present in the slides of whole skin of both species. The epidermal surface of the human skin in which the first 15  $\mu\text{m}$  removed was much more smooth than the intact skin. Parts of the 15  $\mu\text{m}$  sections of the human skin showed good removal of the stratum corneum down to the epidermal layer. Within the wrinkles of the epidermis, some stratum corneum was present where the epidermis and stratum corneum dipped below the level of the microtome blade. Approximately 30 to 50% of the skin had fully intact stratum corneum. This indicates that the blade was not contacting the entire epidermal surface during the initial passes of the microtome. Apparently no stratum corneum was removed from the 15  $\mu\text{m}$  guinea pig skin. Each piece of skin observed had regular ridges along it. Hair follicles were often seen between the ridges. Apparently the microtome did not contact the guinea pig skin when the first section was sliced. The human section which had 100  $\mu\text{m}$  removed showed that most of the epidermis was removed while little of the dermis was removed. Occasionally, some epidermis remained on the dermis between the dermal ridges. On one slide, most of the epidermis was removed

(>80%) while on the other slides, approximately 60 to 80% of the epidermis was removed.

Based on the results of the histological examination, we decided that the stratum corneum and the viable epidermis were not adequately separated. It was decided to combine amounts of lyngbyatoxin A in the stratum corneum and the epidermis into one group called the epidermis.

15. Extraction of Components of Diffusion Apparatus: The glass wool was extracted with 2 ml of chloroform. The tubes were sonicated and shaken to facilitate the extraction of lyngbyatoxin. The extraction procedure was repeated four to five times. Chloroform was added to the receptor fluid and shaken vigorously for several seconds. An emulsion formed which partially separated upon standing in 5 to 10 minutes. The chloroform was drawn off with a pasteur pipet. The receptor fluid was extracted with 10 to 15 ml of chloroform 8 to 12 times; until the aqueous and chloroform phases immediately separated and little if any emulsion formed.

The skin disks were separated into layers by sectioning them parallel to the skin surface with a microtome (Bausch and Lomb Optical Co. Rochester, NY). The blocks containing the frozen skin were mounted in the microtome and adjusted so that the blade would cut parallel to the surface of the skin. The depth adjustment of the microtome was adjusted to 5  $\mu\text{m}$ . The microtome was operated until the blade barely sliced the surface of the skin. Three or four slices were made to remove the first layer of skin (stratum corneum, 15  $\mu\text{m}$ ) which was placed in a scintillation vial. The

microtome blade was wiped with a ball of glass wool dampened with chloroform and then a dry ball of glass wool. The glass wool was placed in the vial with the skin layer. Periodically during the sectioning, liquid freon (Histo Freeze, Fisher Scientific, Pittsburgh, PA) was sprayed on the skin to keep it frozen. The next layer (viable epidermis, 15 to 100  $\mu\text{m}$ ) was sectioned and placed in a vial along with the glass wool used to decontaminate the blade after sectioning. The remainder of the skin was considered to be the dermis. Slices of the dermis 25  $\mu\text{m}$  thick were made until the mounting block was reached so that the skin could more easily be extracted. The mounting block was thawed and the remaining dermis minced with scissors. The dermal slices were added to a vial and the blade decontaminated with glass wool and added to the vial. The skin was extracted with 3 ml of chloroform. The vials were sonicated for five minutes to facilitate extraction of the lyngbyatoxin from the skin. The vials were stored overnight, thawed the next day and the chloroform removed. The minced skin was rinsed with chloroform. Extraction of the skin was repeated for four times.

16. Concentration of the Samples: The chloroform extracts of the skin washes, skin sections and receptor fluids required concentration prior to chromatographic analysis of lyngbyatoxin. An evaporation device was constructed to direct a stream of nitrogen over the samples. Heat was not used to dry the samples because it might have caused break down of lyngbyatoxin. The samples were evaporated to dryness and 200  $\mu\text{l}$  of DMSO added. The use of DMSO as

the solvent had the advantage that volume changes due to evaporation would be reduced. The reconstituted samples were placed in the freezer (-20°C) until they could be diluted and analyzed.

#### Quantitation of Lyngbyatoxin A using HPLC

1. HPLC System: The HPLC system was from Waters Associates (Division of Millipore Corporation Milford, MA) and consisted of a model 510 pump, model 712 Waters Integrated Sample Processor (WISP), model 680 automated gradient controller, model 490 programmable multi-wavelength detector and a model 740 data module. During the first two experiments (using guinea pigs) the detector used was a model 441 fixed wavelength absorbance detector with a model SE-120 strip chart recorder (BBC Goerz Metrawatt Nurnberg, Austria) for recording the data. A Partisil 5  $\mu\text{m}$  RAC II 4.6 mm x 10 cm chromatography column was used (Whatman, Inc. Clifton, NJ).

2. HPLC Conditions: The absorbance detector was set to a 254 nm wavelength and 0.01 absorbance units full scale (AUFS). The basic mobile phase consisted of 85% hexane, 10% chloroform and 5% 2-propanol. All solvents were HPLC grade. After mixing, the mobile phase was filtered through a 0.2  $\mu\text{m}$  47 mm teflon filter (Micron Separations, Inc. Westboro, MA). The flow rate was 0.5 ml/min and the retention time of lyngbyatoxin A was about 9.5 minutes. During the course of the experiments, interfering chromatographic peaks made it necessary to modify the mobile phase. The modified mobile phase was 90% hexane, 6.7% chloroform and 3.3% 2-propanol. At a flow rate of 0.5 ml/min, the modified mobile phase resulted in a

retention time for lyngbyatoxin of about 21 to 24 minutes. When the flow rate was increased to 1.0 ml/min the retention time was about 12.5 to 13.5 minutes.

3. Development of Extraction Method: The chromatographic conditions used to quantify lyngbyatoxin A made it necessary that samples containing lyngbyatoxin A be dissolved in an organic solvent with no water present. It was necessary to develop rapid, sensitive and consistent extraction methods for the aqueous receptor fluid and the skin samples.

a. Solid Phase Extraction: One  $\mu\text{g}$  of lyngbyatoxin was added to five vials containing 2.5 ml of phosphate buffered saline solution (PBSA). Silica solid phase extraction columns were primed with 3 ml of hexane. The samples were aspirated slowly through the columns. Each column was washed with 3 ml of hexane. Lyngbyatoxin A was eluted from the columns with two ml of chloroform. The samples were evaporated under nitrogen, reconstituted in chloroform and injected onto the HPLC. Recovery of lyngbyatoxin A was  $58.2 \pm 16.5\%$  (mean recovery  $\pm$  standard deviation). Recovery of lyngbyatoxin A from reverse phase  $\text{C}_{18}$  and CN columns was below the limit of detection. It was concluded that solid phase extraction columns were not effective for lyngbyatoxin A; therefore, liquid/liquid extraction was evaluated.

b. Liquid/liquid Extraction: Phosphate buffered saline (PBSA, 2.5 ml) containing lyngbyatoxin A (1  $\mu\text{g}$ ) was extracted with methylene chloride, hexane or chloroform. The organic phase was removed and the process was repeated twice. The extracts were

combined, evaporated, reconstituted and quantified by HPLC. The mean recovery of lyngbyatoxin A was  $59.8 \pm 6.3\%$  (n=2) with methylene chloride,  $1.72 \pm 0.5\%$  (n=2) with hexane, and  $69.2 \pm 11.3\%$  (n=2) with chloroform. It was decided that the method of choice for extraction of lyngbyatoxin A from aqueous samples would be liquid/liquid extraction using chloroform as the organic solvent.

4. Stability of Lyngbyatoxin A in Receptor Fluid and Vehicle: Lyngbyatoxin A dissolved in HHBSS (200  $\mu\text{g/ml}$ ) and DMSO (50  $\mu\text{g/ml}$ ) were stored in glass vials at various temperatures and then analyzed. Recovery of lyngbyatoxin A was 89% and 112% after 4 hours of incubation in HHBSS at room temperature (22°C) and 37°C, respectively. Recovery of lyngbyatoxin A from DMSO incubated at -20°C and 22°C for 42 days was 100% and 90%, respectively.

5. Recovery of Lyngbyatoxin A from Diffusion Chambers: The stability of lyngbyatoxin A in the teflon diffusion chambers was studied. Diffusion Chambers were assembled with impermeable teflon discs (Deerfield Scientific Randallstown, MD) in place of the skin discs. The receptor fluid of two diffusion chambers was dosed with 10  $\mu\text{g}$  of lyngbyatoxin A (2  $\mu\text{g}/\mu\text{l}$ ) and incubated at 37°C for 24 hours. Recovery averaged 80.5%. An experiment was done to determine if enzymes leaching out of skin caused breakdown of lyngbyatoxin A. Three diffusion chambers were assembled with human skin and incubated (37°C) for one hour. The skin was removed and replaced by teflon discs. One  $\mu\text{g}$  of lyngbyatoxin A dissolved in DMSO (100

ng/ $\mu$ l) was injected through the sample port into the receptor fluid of each of the three chambers. The chambers were stirred for 1 hour (37°C). The receptor fluid was removed from the chambers and extracted with chloroform. Quantitation of the lyngbyatoxin A in the extracts gave an average recovery of 109.8%  $\pm$  20%. These results indicate that the recovery of lyngbyatoxin A from receptor fluid was effective.

6. Chromatographic Interferences Due to Substances Co-Eluting with Lyngbyatoxin A: During each experiment, control diffusion chambers were assembled with skin but not dosed with lyngbyatoxin A. These chambers were called "blanks" and were used to determine if substances which co-elute with lyngbyatoxin A leach out of the skin. The skin in one diffusion chamber was dosed with 25  $\mu$ l of the vehicle (DMSO), the other chamber received no treatment. The chambers were incubated for either 4 or 24 hours. At the end of the incubation period the blank diffusion chambers were dismantled and their components extracted with chloroform just as described for skin dosed with lyngbyatoxin A. The chloroform extracts from the blank chambers were evaporated under nitrogen, reconstituted with DMSO, diluted and lyngbyatoxin A quantified by HPLC. Interfering substances from the skin extracts were not found except when the extracts were 10 to 50 times more concentrated than normally used. Interfering substances in the blank chambers were also checked for by taking extracts and adding a small amount of lyngbyatoxin A (1:1 5 ng/ $\mu$ l) to the extract. HPLC quantitation showed the presence of the peak corresponding to lyngbyatoxin A in the extract following

addition of lyngbyatoxin A, whereas before addition no lyngbyatoxin A was seen.

A similar procedure was used to determine which peak was lyngbyatoxin A in the samples. A sample was injected into the HPLC and a chromatogram obtained. The same sample was then re-injected after addition of a small amount of lyngbyatoxin A (1:4 5ng/ $\mu$ l). The peak which corresponded to lyngbyatoxin A increased in area while the other peaks decreased in area due to dilution by solvent.

7. Internal Standard: During extractions and sample preparation for HPLC it is beneficial to have an internal standard which can be used to calculate the recovery of the compound of interest (e.g., lyngbyatoxin A). An internal standard is often a closely related compound with chemical properties similar to the compound of interest. Ideally, the retention times and extraction of the two compounds should be similar. Before the extraction is carried out a known amount of the internal standard is added to the sample. Based upon the amount of internal standard recovered, the efficiency of the extraction can be calculated.

Three different compounds were investigated for use as internal standards with lyngbyatoxin A: ethyl 3-indoleacetate, benzimidazole 5-carboxylic acid and indole 5-carboxylic acid (Aldrich Chemical Company, Inc. Milwaukee, WI). Unfortunately, the short retention times of these compounds resulted in interferences by solvent peaks early in the chromatogram. Adjustment of the mobile phase did not sufficiently increase the retention times of the internal standards. It was decided that external standard quantitation would

be used to calculate the amount of lyngbyatoxin A present in the samples.

8. External Standard Quantitation: An external standard indicates that the standard is not in the sample itself but the standard is injected into the HPLC separately from the sample. Also, the standard may be subjected to an extraction procedure prior to quantitation to determine the efficiency of the extraction. The protocol for using external sample quantitation involves first injecting several different amounts of the compound of interest (lyngbyatoxin A) into the HPLC. In each case, the detector response or peak (ultraviolet absorption versus time) is recorded and the area under the curve is calculated by means of an integrator. Beer's law states that the absorbance of the substance is proportional to the concentration of the substance in the sample provided that deviations from ideal behavior do not occur (Skoog and West, 1979). Applied to this situation, Beer's law says that the area of the standard peak should be linearly related to the amount of the standard injected into the HPLC. Nine different amounts of lyngbyatoxin A (ranging from 2.5 ng to 500 ng) were injected into the HPLC. The detector response (area) was linearly related to the amount of lyngbyatoxin A injected, with a correlation coefficient of 0.99895 (see Figure 5).

During the experiments, two different amounts of lyngbyatoxin A were used as standards. Linear regression was used to determine the amount of lyngbyatoxin A present in the samples based upon the area of the lyngbyatoxin A peak. Also, the response factor (ng /

peak area) was calculated for standards. The response factors were averaged from multiple injections of standards with the same amount of lyngbyatoxin A. Comparison of the response factors for fresh standards were made and reduced errors in quantitation due to inaccurate standards. Samples containing amounts of lyngbyatoxin A within the linear portion of the detector response curve were calculated by using the following formula:

$$\text{Sample Amount} = \text{Sample Peak Area} \times \text{Response Factor}$$

#### **Determination of Partition Coefficient of Lyngbyatoxin A and [<sup>3</sup>H]PbTx-3**

In most cases the partition coefficient is easily determined by dissolving a known quantity of the solute in a flask containing water and an immiscible organic solvent and shaking until equilibrium is reached, usually within five to ten minutes. The phases are allowed to separate and/or the flask centrifuged to break the emulsion. The phases are then analyzed for the solute. Usually it is sufficient to measure the solute in only one phase and determine the quantity in the other phase by subtraction. If the compound adheres to the walls of the flask or interacts in some other way, both phases must be measured (Leo et al., 1971). Care must be taken not to contaminate one phase with the other when sampling (Riley, Ronald T. USDA, Athens, GA. Personal Communication).

The octanol/water partition coefficient (log P) for lyngbyatoxin A and [<sup>3</sup>H]PbTx-3 was determined. The latter compound is a lipophilic, low molecular weight (896 g/mol) algal toxin which

is also being used in skin penetration studies in our laboratory. The chloroform/water partition coefficient for lyngbyatoxin A could not be determined because the lyngbyatoxin A was not sufficiently soluble in the water phase to cause a decrease in the amount lyngbyatoxin A in the chloroform phase.

Validation of the method was done by determining the partition coefficient of benzoic acid and toluene and comparing the results to published values.

1. [ $^{14}\text{C}$ ]-benzoic Acid: The [ $^{14}\text{C}$ ]-benzoic acid (specific activity 21.8 mCi/mmol, NEN Research Products, Boston, MA) was reconstituted with 2 ml of distilled water. The stock solution was filtered with a 0.2  $\mu\text{m}$  filter (Acrodisc, Gelman Sciences, Ann Arbor, MI). The concentration of the stock solution (1.495  $\mu\text{g}/\mu\text{l}$  or 12.25 nmol/ $\mu\text{l}$ ) was determined using standard liquid scintillation (LSC) techniques and a Packard Model 1500 Tri-Carb Liquid Scintillation Analyzer, (Packard Instrument Co., Sterling, VA). A portion (60 ng/40  $\mu\text{l}$ ) of the stock solution was added to distilled water (total volume 100  $\mu\text{l}$ ) and placed in a polypropylene microcentrifuge tube with 100  $\mu\text{l}$  of 1-octanol (Fisher Certified, Fisher Scientific Co., Norcross, GA). The determinations were done in quadruplicate. The tubes were shaken in a Roto-Torque rotator (Cole Parmer, Chicago, IL) at room temperature (22°C) for 24 hours. The tubes were then centrifuged at approximately 1500Xg for 30 minutes to separate the phases. Aliquots of each phase were analyzed with standard LSC techniques.

2. [ $^{14}\text{C}$ ]-toluene: The [ $7\text{-}^{14}\text{C}$ ]-Toluene, (NEN Research Products) had

a specific activity of  $2.01 \times 10^{-2}$  uCi/mmol.  $^{14}\text{C}$ -toluene (43 ng/50  $\mu\text{l}$ ) was added to 1 ml of 1-octanol and partitioned with 5 ml of distilled water. A larger volume of water than octanol was required because most of the toluene distributed into the octanol phase leaving little toluene in the water phase to be detected. The partition coefficient is dependent on the concentrations of the compound of interest in each phase, not the volumes of the phases. The partition coefficients were done in quadruplicate. The vials were shaken for 20 minutes at room temperature and then centrifuged for 30 minutes. Radioactivity in the water and octanol phases was analyzed by LSC in triplicate.

3. [ $^3\text{H}$ ]PbTx-3: A Waters Associates HPLC system (Milford, MA) was used to determine the radiochemical purity of [ $^3\text{H}$ ]PbTx-3 in the stock solution and in the water and organic phases at the termination of the partition experiment. The HPLC detector was a Flo- One/Beta radioactive flow detector (Radiomatic Instruments and Chemical Co., Tampa, FL). A Whatman Partisphere  $\text{C}_{18}$  5 micron cartridge column, (Whatman, Clifton, NJ) was used. The mobile phase consisted of 85% methanol, 15%  $\text{H}_2\text{O}$  with a flow rate of 1 ml/min. The stock [ $^{42-3}\text{H}$ ]PbTx-3 (radiochemical purity >99%, 9.93 Ci/mmol), was obtained from Dr. Baden (University of Miami, Miami, FL), and was determined to have a concentration of 39 nmol/ml or 35.3 ng/ $\mu\text{l}$ . Stock [ $^3\text{H}$ ]PbTx-3 (883 ng/25  $\mu\text{l}$ ) was added to a vial, evaporated under a stream of nitrogen and 1 ml each of octanol and distilled water added. The vials were prepared in quadruplicate and shaken at room temperature for 15 min. The vials were centrifuged for 15 min

and each phase analyzed by LSC and HPLC. The HPLC analysis showed that PbTx-3 is unstable in 1-octanol. Therefore, it was necessary to determine the partition coefficient for PbTx-3 in chloroform/water by using similar methods. HPLC analysis showed that PbTx-3 is stable in this system. Stock [<sup>3</sup>H]PbTx-3 (1412 ng/40  $\mu$ l) was added to a vial, dried down, dissolved in 1 ml of chloroform (Fisher Certified), and 1 ml distilled water. Duplicate vials were shaken for 15 min and then centrifuged. Each phase was analyzed by LSC and HPLC techniques.

4. Lyngbyatoxin A: Two octanol/water partition coefficient determinations for lyngbyatoxin A were made. In the first experiment a vial containing 400 ng lyngbyatoxin A in 1-octanol (200  $\mu$ l) and six ml of deionized water was shaken in the rotary shaker for 20 min. The temperature was 22°C. The vial was centrifuged at 1500Xg for 20 min to separate the two phases. An aliquot of the octanol phase was removed, diluted 1:2 with chloroform and quantified using the HPLC. The standard used was an aliquot of the octanol solution containing lyngbyatoxin A (2 ng/ $\mu$ l) diluted 1:2 with chloroform. Injections of standard and unknowns were run in duplicate in the first trial. The second experiment was run in duplicate with a slight modification of the procedure. Two vials containing 400 ng lyngbyatoxin A in octanol (200  $\mu$ l) and 10 ml of water were mixed in the rotary shaker for 20 min. The vials were centrifuged and aliquots of the octanol phase analyzed for lyngbyatoxin A. Injections were made in triplicate.

The partition coefficient (Log P) was calculated as follows:

$$\text{Log P} = \text{Log} (C_{\text{octanol}}/C_{\text{water}})$$

Where C is the concentration of lyngbyatoxin A in the respective phase.

#### **Statistical Treatment of the Data**

Statistical analysis of the data was done using PCANOVA statistical analysis software (Human Systems Dynamics, Northridge, CA) and Statgraphics statistical graphics system (Statistical Graphics Corporation, Rockville, MD). The amount of lyngbyatoxin A found in the dermis and the receptor fluid of each diffusion chamber was summed (this sum was denoted as "skin penetration") and analyzed statistically. The amount of lyngbyatoxin found in the skin wash, stratum corneum and epidermis were tabulated to determine total recovery but were not subjected for statistical analysis.

Single factor with repeated measures (over time) analysis of variance (ANOVA) was performed on the skin penetration of the guinea pig and human data separately. Statistical analysis (ANOVA) of skin penetration showed that there were no significant differences between the different time periods for the guinea pig and for the human.

The variances of the skin penetration in the guinea pig and human were tested to see if they were significantly different (as described by Zar (1984)). The ratio of the variances was 10.336, the

value of  $F_{(0.05(2), 18, 25)}$  was 2.34; therefore, the variances were significantly different. Because the two populations (guinea pig and human data) did not have the same variance, a modified t test was used to test for differences between the guinea pig and the human skin penetration at each time period. The test used was "Welch's approximate t" (Zar, 1984; Steel and Torrie, 1980). The means and variances were calculated using the STATGRAPH computer program.

## RESULTS AND DISCUSSION

### **Skin Penetration Experiments**

Results of the skin penetration are shown in Table 2 and 3 and are graphed in Figures 6, 7 and 8. The amount of lyngbyatoxin A measured in the dermis and the receptor fluid was summed in order to calculate in vitro skin penetration. The statistical analysis showed that there were no significant differences between the different time periods for the guinea pig or the human skin penetration data. T-tests performed to compare the human and the guinea pig skin penetration showed statistically significant differences ( $p \leq 0.05$ ) between each mean at each time period. Figure 8 and Table 3 illustrate the difference in skin penetration of the guinea pig and the human at each time period. The amounts of lyngbyatoxin A present in the dermis and receptor fluid of the guinea pig were:  $25 \pm 7.4\%$ ,  $25 \pm 4.6\%$ ,  $29 \pm 3.3\%$  and  $23 \pm 3.0\%$  (mean  $\pm$  SE) at the 1, 4, 24 and 48 hr time periods, respectively. The amounts in the dermis and receptor fluid of the human were: 5.7

$\pm 1.3\%$ ,  $5.2 \pm 1.1\%$ , and  $7.2 \pm 1.4\%$  at the 1, 4 and 24 hr time periods, respectively. Skin penetration of lyngbyatoxin A in the guinea pig was 4.3, 4.9, and 3.9 times more than in the human at 1, 4, and 24 hr.

The results of recovery experiments from guinea pig and human skin ( $65 \pm 13\%$  for the guinea pig, and  $66\% \pm 18\%$  for the human) demonstrate that extraction of lyngbyatoxin A from the skin could be incomplete. There was little difference in recovery of lyngbyatoxin A from the skin of the two species. Recovery of lyngbyatoxin A from the components of the skin penetration chambers was good. Average recoveries ranged from  $83 \pm 3.7\%$ ,  $\pm 10\%$ , and  $84 \pm 2.4\%$  for the guinea pig at the 1, 4, and 24 hr incubation times, respectively. Recoveries ranged from  $85 \pm 4.1\%$ ,  $82 \pm 3.9\%$ , and  $86 \pm 2.3\%$  for the human at the 1, 4, and 24 hr incubation times, respectively. It seems likely that the unrecovered lyngbyatoxin A in these experiments could be accounted for by assuming that the skin contained some un-extracted lyngbyatoxin A.

In vitro skin penetration of lyngbyatoxin A dissolved in DMSO occurs rapidly in both human and guinea pig skin. The percent of the total dose of lyngbyatoxin A recovered from the epidermis was 12% in the guinea pig and 17% in the human at 1 hr. Furthermore, lyngbyatoxin A was present in the dermis of both species (5.7% of the total dose in human and 25% in guinea pig). The rapid skin penetration of the lipophilic lyngbyatoxin A is consistent with the results of the partition coefficient determination.

The ANOVA showed no statistically significant changes in skin penetration over time. The lack of significant change over time

indicates there was rapid penetration of lyngbyatoxin A within the first hour and little change thereafter. It is known that during the very early time course of penetration, the trans-appendageal route of skin penetration is important; but, diffusion through the stratum corneum predominates during steady state diffusion (Scheuplein, 1967). It is unlikely that the transappendageal skin penetration route is the only major route during the one hour time period because the area available for transappendageal absorption is only a small fraction (less than 0.2% including sweat glands) of the total skin area for the human being (Scheuplein, 1967). Even if it is assumed that the guinea pig has twice as much total follicular area as a rat (Bronaugh et al., 1982), the percentage of skin area of dermal appendages would only be about 0.3%.

Undoubtedly an important factor in the amount of percutaneous penetration of lyngbyatoxin A is the selection of the vehicle. The vehicle used in this study, DMSO, is a potent penetration enhancer. In vivo, the enhancement effect appears to be reversible, but in vitro it appears to be permanent (Embery and Dugard, 1971, Malkinson and Gehlmann, 1977). At high concentrations, DMSO has been reported to displace water from the skin, denature proteins, extract lipids and damage cells (Embery and Dugard, 1971; Malkinson and Gehlmann, 1977). Visual inspection of the skin following the experiments did not disclose any damage. It is likely that DMSO caused irreversible microscopic or molecular changes in the skin which permitted a large amount of lyngbyatoxin to penetrate through the stratum corneum and the viable epidermis to the dermis below.

The amount of lyngbyatoxin A actually penetrating into the

receptor fluid was extremely small, even at the later time periods. The maximum percentage of lyngbyatoxin A found in the receptor fluid was 0.38% of the total dose in one of the chambers. Often no lyngbyatoxin A was detected in the receptor fluid. Recovery of lyngbyatoxin A from receptor fluid was greater than 80%. Lyngbyatoxin A apparently partitions into the dermis which is more lipophilic than the receptor fluid. In a living animal, the dermal vasculature carries penetrants from the dermis to systemic circulation and maintains the concentration gradient between the epidermis and dermis. The excised skin lacks a functional capillary circulation and this leads to a build-up of the lipophilic lyngbyatoxin in the dermis (Hawkins and Reifenrath, 1984; Hawkins and Reifenrath, 1986).

Figure 8 illustrates the significant difference in the skin penetration of lyngbyatoxin A in the guinea pig and the human. Many investigators have shown that the principal barrier to percutaneous absorption is the stratum corneum (Blank, 1965; Scheuplein, 1965; Idson, 1975; Tojo et al., 1987). There is no evidence that guinea pig and human dermis or epidermis differ in such a way as to cause the difference in permeability of lyngbyatoxin A. Laboratory rodents, however, have a thinner stratum corneum than humans (Kligman, 1964; Bronaugh et al., 1982). Holbrook and Odland concluded that in man, the rates of absorption of hydrocortisone or pesticides were inversely related to stratum corneum thickness (Holbrook and Odland, 1974). Scheuplein and Bronaugh (1983) and Bronaugh et al. (1982) have shown that the thickness of the stratum corneum contributes to species

differences. Other authors believe that the lipid composition of the stratum corneum is more important in determining the percutaneous penetration of substances (Elias et al., 1981). We believe that the combination of thinner stratum corneum and more hair follicles are responsible for the three to four times greater skin penetration of lyngbyatoxin A in the guinea pig compared to the human.

#### **Estimate of Absorbed Dose Under Hypothetical Conditions**

The skin penetration results have been used to estimate the dose absorbed from topical exposure to a solution of lyngbyatoxin A dissolved in DMSO. The following assumptions were made for these calculations: Firstly, the results of the in vitro study correlate with in vivo skin penetration. Several studies have demonstrated the validity of this assumption (Hawkins and Reifenrath, 1986; Kempainen et al., In Press). Secondly, the minimum lethal dose in man is the same as in the mouse ( $LD_{100} = 0.03$  mg/kg I.P.). Thirdly, the same degree of toxicity results from epidermal as parenteral exposure provided the amount of toxin entering the body is the same. This assumption is likely to be true if the metabolism and/or elimination of lyngbyatoxin A is the same by both routes of administration. Fourthly, skin of the abdominal region is 1.75 times more permeable than skin on the hand. It is assumed that the hand has similar permeability characteristics to flexor forearm skin. The abdomen is 2.1 times more permeable to parathion than abdominal skin and 1.4 times more permeable to malathion (Holbrook and Odland, 1974). The mean of 2.1 and 1.4 is 1.75. The back of the hand was found to be 1.65 times less permeable to water than

abdominal skin when the flux of transepidermal water loss was compared (Scheuplein and Bronaugh, 1983).

The hypothetical situation involves an adult human weighing 70 kg is exposed to a solution of lyngbyatoxin A at a concentration similar to that used in this study ( $25.77 \mu\text{g}/12.9\mu\text{l DMSO}/\text{cm}^2$  skin surface). The man is exposed for an hour over the entire surface of his hands (surface area of  $860 \text{ cm}^2$ ) (Kemppainen et al., 1988-1989). It is further assumed that if 5.7% of the dose is absorbed through abdominal skin in 1 hour (as described in this study), then 3.26% of the dose is absorbed through the hands ( $5.7\% \times (1/1.75)$ ) in an hour which is  $0.839 \mu\text{g}/\text{cm}^2$ . The total amount of lyngbyatoxin A absorbed over the entire surface of the hands is  $0.839 \mu\text{g}/\text{cm}^2 \times 860 \text{ cm}^2 = 721.5 \mu\text{g}$ . The amount of lyngbyatoxin absorbed per kilogram of body weight is  $721.5 \mu\text{g}/70 \text{ kg} = 10.3 \mu\text{g}/\text{kg}$ . The  $\text{LD}_{100}$  i.p. in mice is  $30 \mu\text{g}/\text{kg}$  or only 3 times higher. The minimum dose of lyngbyatoxin A required to cause more subtle toxic effects is not known. Lyngbyatoxin A is a potent inflammatory agent and severe burns and blisters on the hands would undoubtedly result.

#### Chromatographic Analysis of Samples

Chromatograms were compared in an effort to identify peaks associated with breakdown or metabolism of lyngbyatoxin A. This was done by looking for changes in the chromatograms which could be correlated with treatment or time. All chromatograms from skin extracts or lyngbyatoxin A standards had large solvent front peaks from approximately 2 to 4 minutes in retention time. Chromatograms

of control skin extracts (including skin treated with 25  $\mu$ l of DMSO) contained early eluting peaks (4 to 8 minutes) which were usually small in size. Chromatograms from lyngbyatoxin A treated skin also had the early eluting peaks which varied considerably in size but were generally larger than the same peaks found in the control skin extracts. Figure 9 shows an example of chromatograms from a single human skin penetration experiment. In the Figure, the size of certain late appearing peaks increased during longer incubation periods; however, this trend was not consistently observed. The identification of these substances was not made. It is possible that these substances were lyngbyatoxin A metabolites or breakdown products or substances released by the skin in response to lyngbyatoxin A. While it is known that DMSO can extract lipoproteins and nucleoproteins from the skin (Embery and Dugard, 1971), it is believed that the substances appearing in the chromatograms were not released in response to DMSO alone. A comparison of chromatograms from control skin and control skin treated with DMSO alone failed to show any differences. As previously mentioned, substances with retention times longer than lyngbyatoxin A (for instance, the peak just after lyngbyatoxin A of the 24 hr sample in Figure 9) were not well correlated with lyngbyatoxin A treatment.

#### **Partition Coefficient Determination**

1. [ $^{14}\text{C}$ ]-Benzoic Acid: The average of four measurements of the octanol/water partition coefficient ( $\text{Log } P_{\text{octanol/water}}$ ) of  $^{14}\text{C}$ -benzoic acid was  $1.25 \pm 0.06$  (mean  $\pm$  standard error). The

literature value for benzoic acid was 1.87. However, the published method used an aqueous phase with a pH of 7.4 and included hexadecylamine (Leo et al., 1971). The difference in pH and composition of the method used and the published method probably accounts for the difference in results. The pH of a solution may affect the partition coefficients of acid and bases (Leo et al., 1971).

2. [14C]-Toluene: The experimentally determined the  $\text{Log}P_{\text{Octanol/water}}$  of  $^{14}\text{C}$ -toluene was determined to be  $2.54 \pm 0.05$ . The literature values were: 2.69, 2.73, 2.11 and 2.80 (Leo et al., 1971). The experimental results agreed with the published values. The method used in this study was judged to be accurate.

3. [ $^3\text{H}$ ]PbTx-3: The chloroform/water partition coefficient for [ $^3\text{H}$ ]PbTx-3 was determined. The  $\text{log } P_{\text{CHCl}_3/\text{water}}$  value was  $2.20 \pm 0.05$  ( $n=2$ ). Leo and coworkers (Leo et al., 1971) have derived linear equations relating the Log P values in various solvents to the Log P values in a reference system which is 1-octanol/water. They group solutes into two general classes, hydrogen donors which include acids, phenols, alcohols, etc. The other group includes hydrogen acceptors such as aromatic hydrocarbons, ethers, esters, ketones and others. The solvent chloroform is a weak H-donor and causes many of the solutes in the hydrogen donor group (e.g., barbiturates, alcohols, imides and amides among others) to be neutral in character. PbTx-3 is a polycyclic ether. This would place PbTx-3 in the hydrogen acceptor group.

The linear equation for the  $\text{CHCl}_3$  system with H-acceptor solutes is:

$$\text{Log } P_{\text{CHCl}_3/\text{water}} = 1.276 \text{ Log } P_{\text{octanol}/\text{water}} + 0.171$$

Based on our data ( $\text{Log } P_{\text{CHCl}_3/\text{water}} = 2.20$ ), the calculated  $\text{Log } P_{\text{octanol}/\text{water}}$  for [ $^3\text{H}$ ]PbTx-3 is 1.59.

4. Lyngbyatoxin A: An average of three measurements gave a mean  $\text{Log } P_{\text{octanol}/\text{water}}$  of  $1.53 \pm 0.020$  for lyngbyatoxin A. This value indicates that Lyngbyatoxin A is 33.88 times more soluble in octanol than in water. Increasing the solubility of a compound in the stratum corneum or epidermis (by increasing the partition coefficient) increases the rate of skin penetration of steroids (Tojo et al., 1987), organic alcohols (Scheuplein, 1965), benzoic acid, acetylsalicylic acid and urea (Bronaugh et al., 1982). However, it has been proposed that partition coefficients near one favor skin penetration by allowing the compound to dissolve in both the aqueous and lipid phases of the skin. Esters of betamethasone with partition coefficients nearest to one had the highest topical activity (Idson, 1975). Higuchi reported that highly lipid soluble compounds would have a low affinity for the more aqueous deeper layers of the skin and exhibit low rates of skin penetration (Higuchi, 1960). The partition coefficient for lyngbyatoxin A indicates that lyngbyatoxin A could readily be absorbed through skin. The fact that solutions of lyngbyatoxin A applied to mouse ears causes inflammation and promote tumors (Fujiki et al., 1981;

Fujiki et al., 1983) demonstrates that lyngbyatoxin A penetrates skin in vivo.

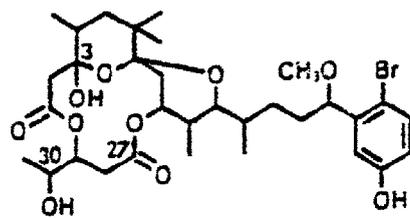
#### CONCLUSIONS

It is important to determine the amount of skin absorption which occurs during topical exposure to lyngbyatoxin A because people are exposed to this toxin when swimming in marine water containing blue-green algae or when studying this compound in research laboratories. The results of this study indicate lyngbyatoxin A readily penetrates into human and guinea pig skin during short periods of exposure (1 hour) when the vehicle is DMSO and guinea pig skin is about four times more permeable to lyngbyatoxin A than human skin when the vehicle is DMSO.

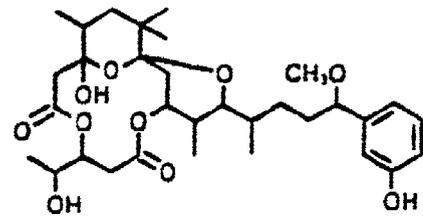
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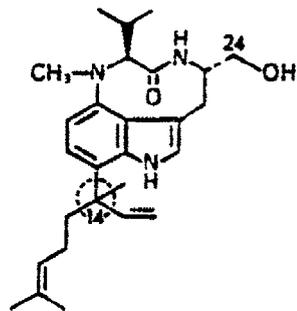
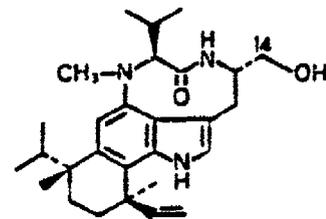
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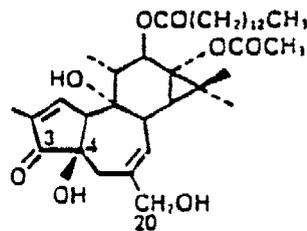
Aplysiatoxin



Debromoaplysiatoxin

Teleocidin A  
Lyngbyatoxin A

Teleocidin B



TPA

Figure 1. Structures of tumor promoters: (a) Aplysiatoxin, (b) Debromoaplysiatoxin, (c) Lyngbyatoxin A, (d) Teleocidin B, (e) 12-O-tetradecanoylphorbol-13-acetate (TPA).

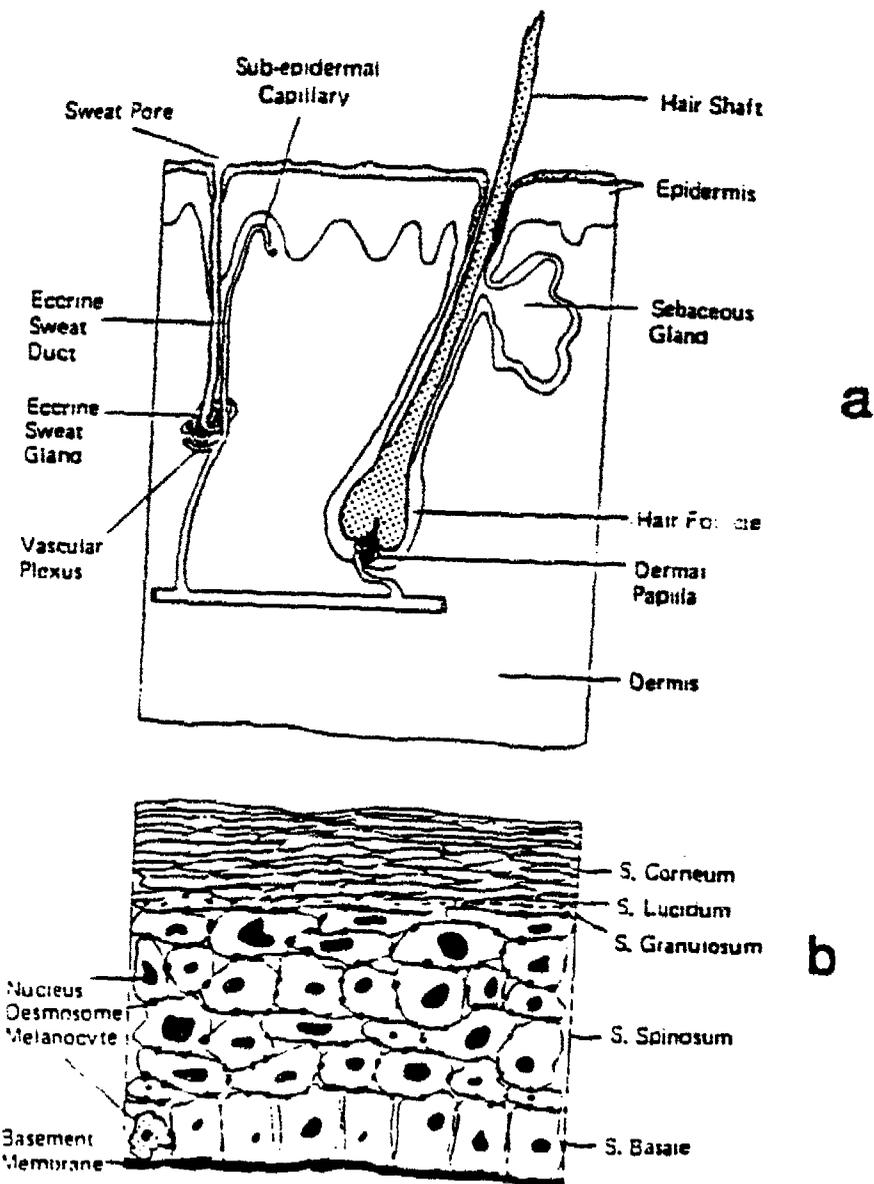


Figure 2. A simplified diagram of the human skin: (a) epidermis, dermis, and cutaneous appendages including blood supply, (b) layers of the epidermis. Reproduced with permission from the Annual Review of Medicine Vol. 39, copyright 1988 by Annual Reviews, Inc.

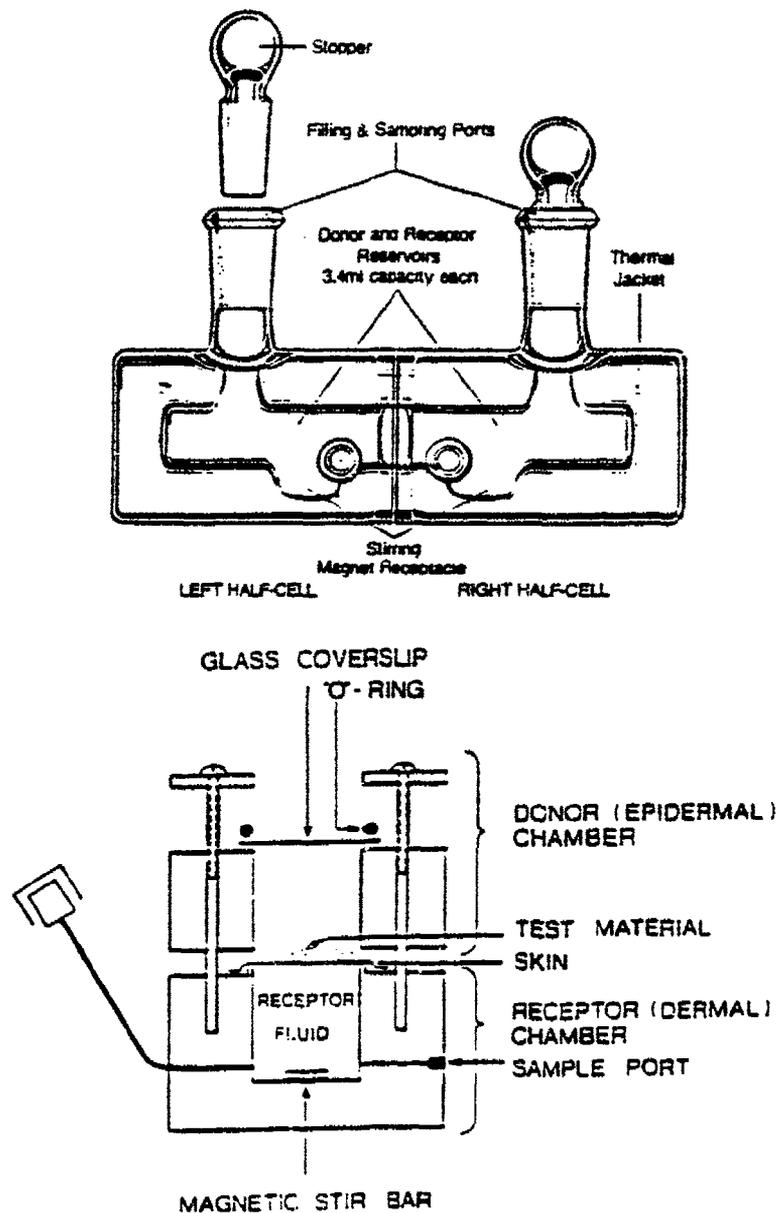


Figure 3. (a) Example of an infinite dose diffusion chamber. Reproduced with permission of Vanguard International, Neptune, NJ. (b) Static finite dose diffusion chamber used in this study. Reproduced with permission from Toxicol, Vol. 25, copyright 1987.

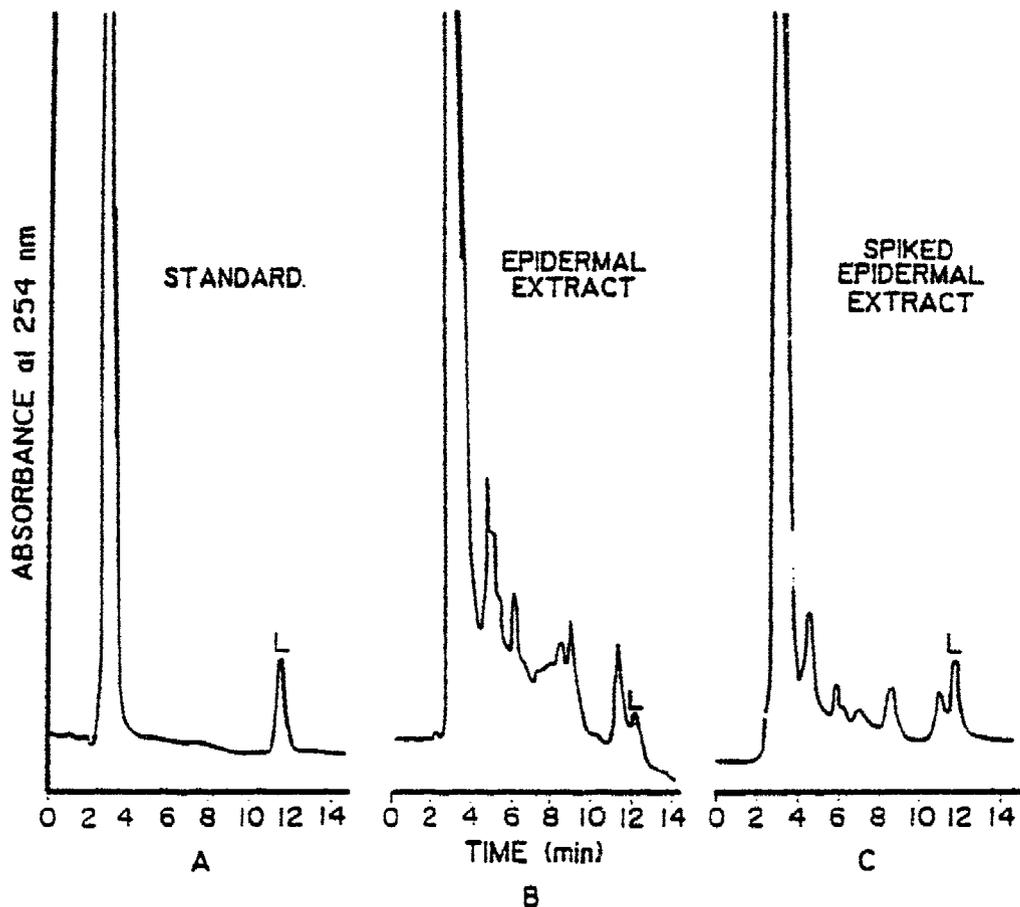


Figure 4. Identification of lyngbyatoxin A in samples: (A) Lyngbyatoxin A standard, 50 ng/10  $\mu$ l injection (L is lyngbyatoxin A). (B) Epidermal extract, 50  $\mu$ l, 1:10 dilution of original 200  $\mu$ l sample is injected (L is prospective lyngbyatoxin A). (C) 25  $\mu$ l of the 5 ng/  $\mu$ l standard is added to 75  $\mu$ l of epidermal extract. 50  $\mu$ l is injected. The peak corresponding to lyngbyatoxin A increases in size. Mobile phase: 85% hexane, 10% chloroform, 5% 2-propanol. Flow rate: 0.5 ml/ min, detector: 0.01 AUFS. Standard peak is approximately 0.0005 absorbance units.

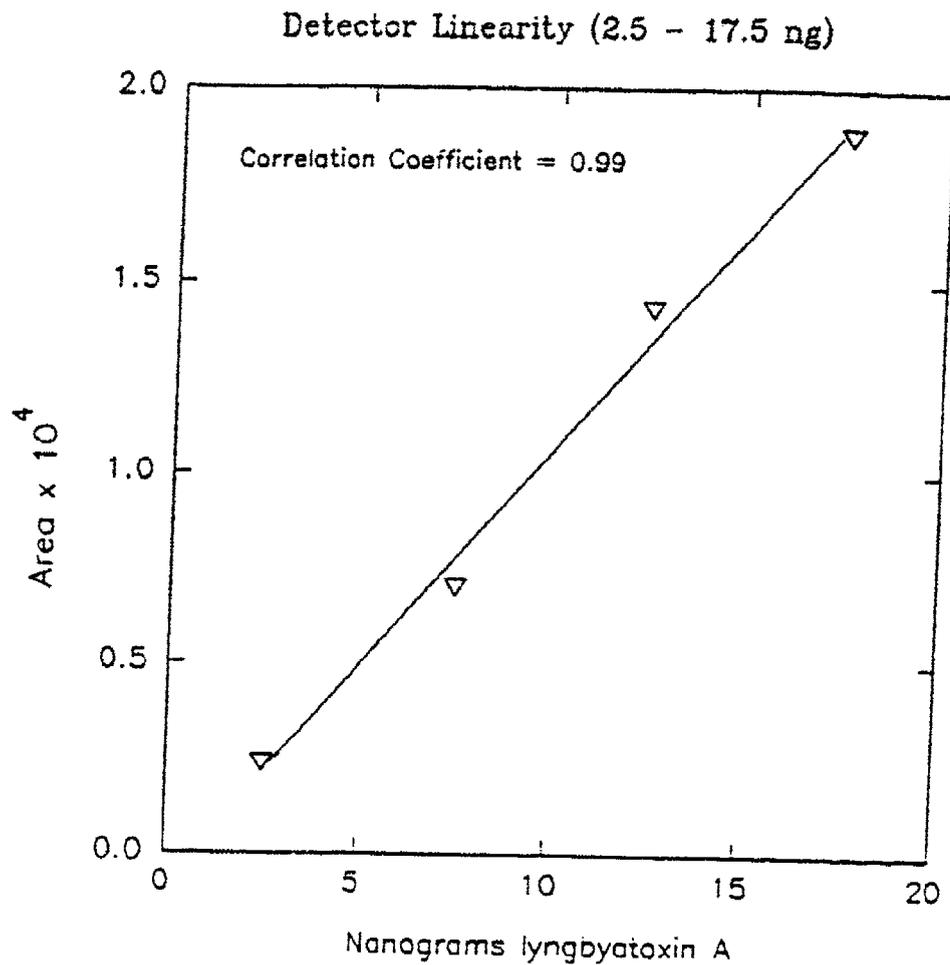


Figure 5. Linear relationship between amount of lyngbyatoxin A injected (2.5 - 17.5 ng) and detector output expressed as peak area. Mobile phase: 85% hexane, 10% chloroform, 5% 2-propanol. Detector set at 0.01 AUFS. Amounts detected in experiments typically ranged from 5 to 150 ng.

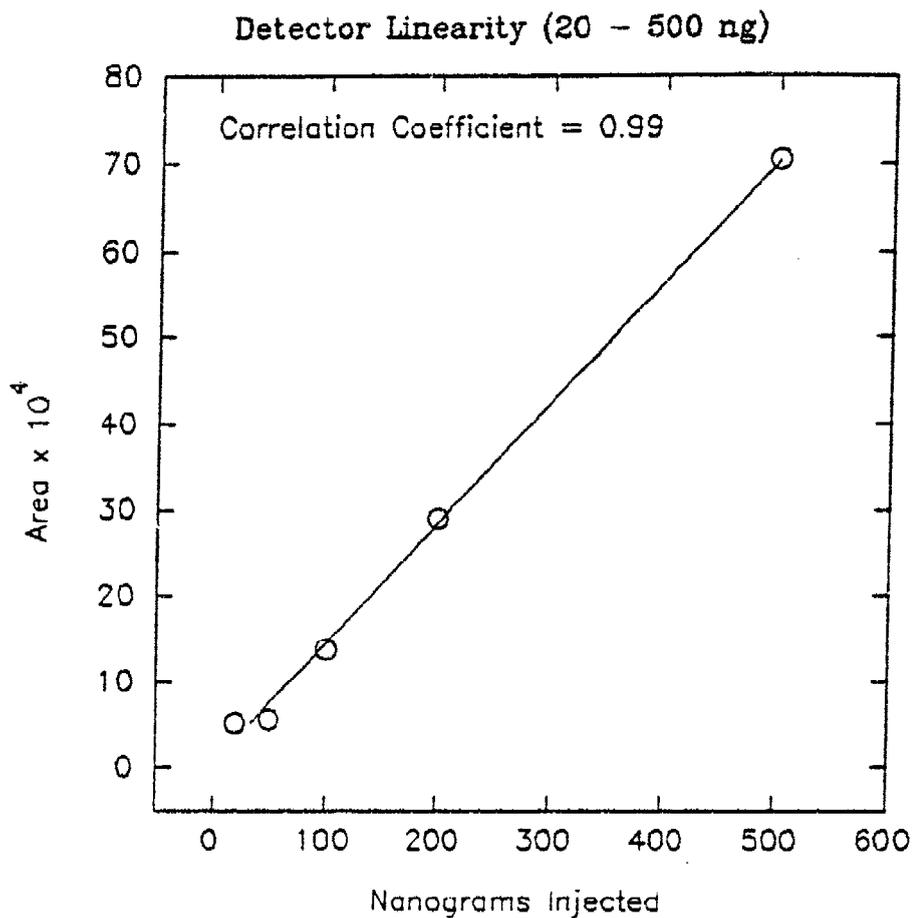


Figure 6. Linear relationship between amount of lyngbyatoxin A injected (20 - 500 ng) and detector output expressed as peak area. Mobile phase was 85% hexane, 10% chloroform, 5% 2-propanol. Detector set at 0.01 AUFS. Amounts detected in experiments typically ranged from 5 to 150 ng.

## Amount of Lyngbyatoxin A on Skin Surface

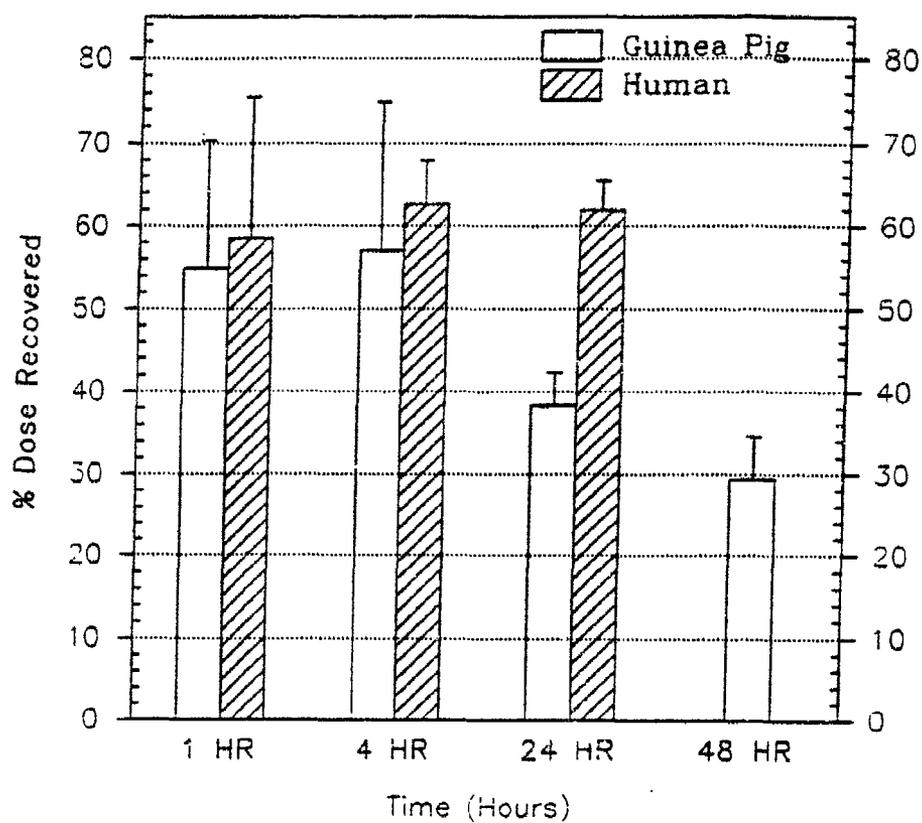


Figure 7. Lyngbyatoxin A recovered from the surface of the skin at the end of each incubation time. The results are expressed as mean  $\pm$  standard error (total dose: 50  $\mu$ g).

## Amount of Lyngbyatoxin A in Epidermis

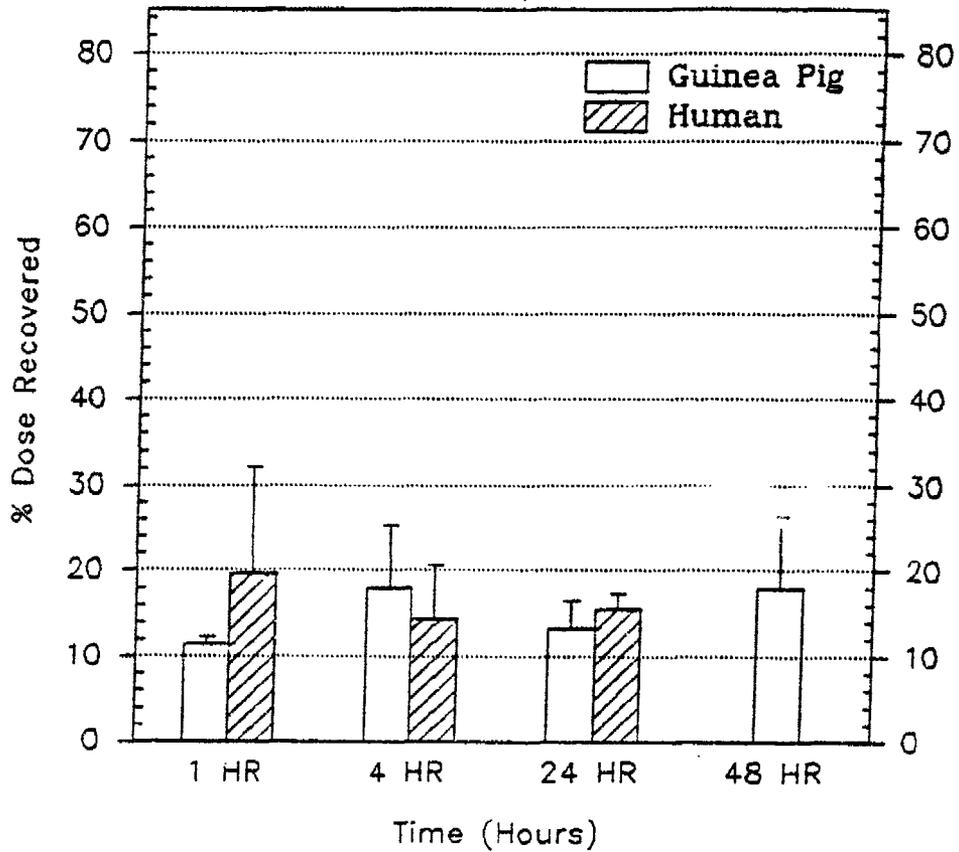


Figure 8. Lyngbyatoxin A recovered from the epidermis at the end of each incubation time. The results are expressed as mean  $\pm$  standard error (total dose: 50  $\mu$ g).

## Amount of Lyngbyatoxin A in Dermis &amp; Receptor Fluid

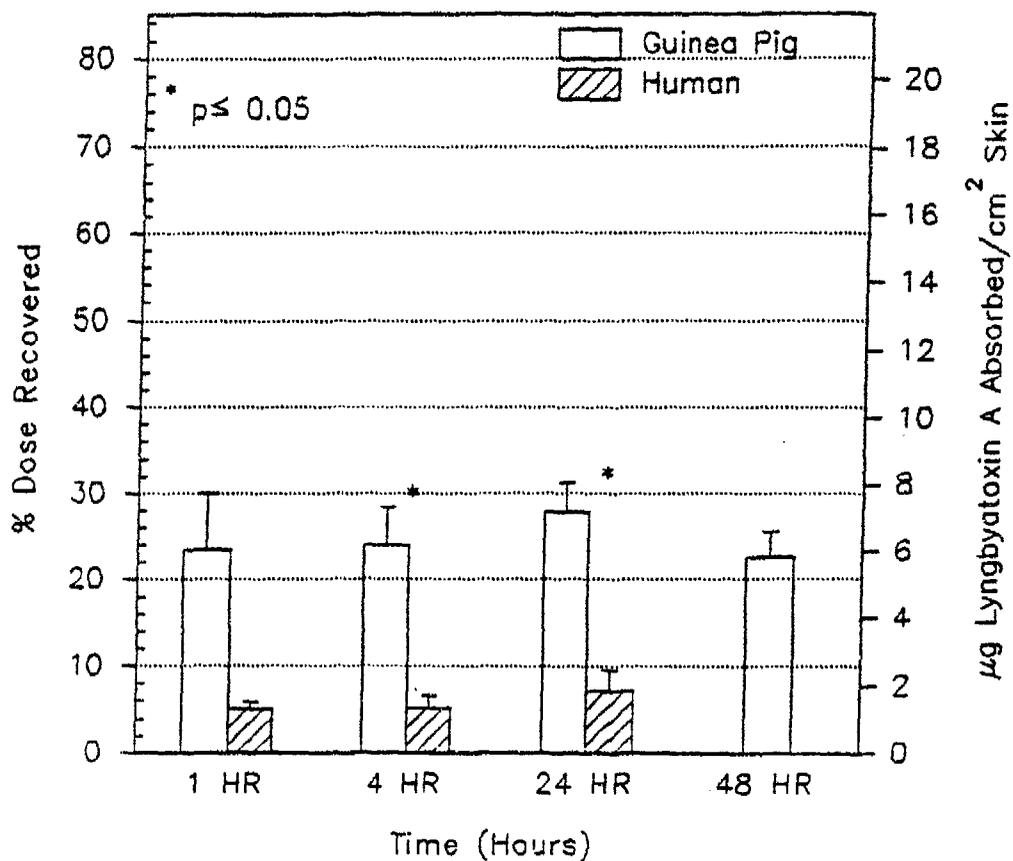


Figure 9. *In vitro* skin penetration of amount of lyngbyatoxin A based on recovery of lyngbyatoxin A from the dermis and receptor fluid at the end of each incubation time. The results are expressed as mean  $\pm$  standard error. The asterisk indicates significant difference of the mean skin penetration of the guinea pig and the human. At the 1 hr time period the means were not significantly different.

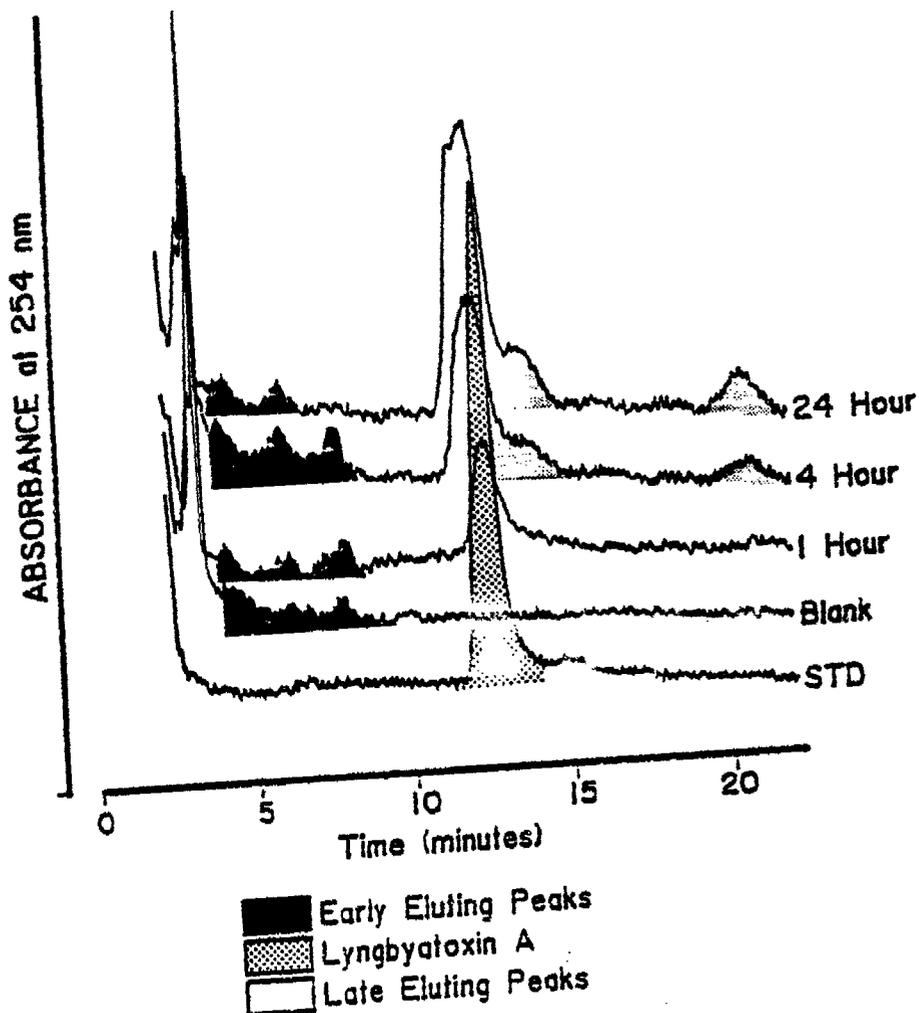


Figure 10. Comparison of chromatograms for possible lyngbyatoxin A metabolites. The different chromatograms are superimposed and are from one human skin penetration experiment. The lyngbyatoxin A standard injected was 225 ng ("STD" in the figure). A control skin extract was not treated with lyngbyatoxin A ("Blank"). The chromatograms labeled 1, 4, and 24 hours are the incubation times of the human skin treated with 50  $\mu$ g lyngbyatoxin A. Mobile phase: 90% Hexane, 6  $\frac{2}{3}$ % chloroform, 3  $\frac{1}{3}$ % 2-propanol. Detector: 0.01 AUFS. The standard is approximately 0.0014 absorbance units.

**PENETRATION OF MICROCYSTIN (A BLUE GREEN ALGAE TOXIN) THROUGH  
EXCISED MOUSE, GUINEA PIG AND HUMAN SKIN**

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**STATEMENT OF PROBLEM**

The purpose of this study was to determine if microcystin can penetrate excised human skin. The degree of correlation between in vivo and in vitro cutaneous absorption of compounds depends on the compounds, species and methodology used in investigations (Bronaugh, Stewart, Congdon et al. 1982). In many cases there is a good correlation between in vivo and in vitro penetration (Kemppainen, Pace and Riley, 1987). The specific objectives of the present study were to determine: 1) the extent to which microcystin penetrates excised human skin, 2) if excised mouse or guinea pig skin is the better model for human skin, 3) the effect of vehicle [water, DMSO and methanol] on penetration of microcystin through human skin and 4) the effect of dose of microcystin on penetration through guinea pig skin.

## BACKGROUND

Microcystin is a blue green algae (cyanobacteria) toxin produced by some strains of Microcystis aeruginosa (Carmichael, Jones, Mahmood and Theiss, 1985a; Watanabe and Oishi, 1980). Several types microcystins have been isolated i.e. microcystin-LR, YR and RR, also called Cyanoginosins-LR, YR and RR, respectively (Botes, Tuinman, Wessels et al., 1984; Botes, Wessels, Kruger et al., 1985). Microcystin-LR is the principal toxic peptide produced by laboratory isolate of M. aeruginosa. This toxin is a cyclic heptapeptide (Fig.1) containing several unusual amino acids and has a molecular weight of 994 daltons (Botes et al., 1984; Krishnamurthy, Carmichael and Sarver, 1986). Toxic blooms of several strains of blue green algae are found worldwide in both natural and man made fresh water lakes (Carmichael, 1981). The most favorable conditions for a bloom to occur are warm, dry, low wind days of summer and early fall. Increased pollution in urban, recreational and agricultural water sources seems to contribute to the growth of toxic and nontoxic blooms (Skulberg, Codd and Carmichael, 1984). Consumption of toxins in water and bloom mass have been implicated in the loss of live stock and wild animals in several countries throughout the world (Carmichael et al. 1985a; Skulberg et al. 1984; Beasley, Coppock, Simm et al. 1983), as well as human intoxication (Billings, 1982; Falconer, Beresford and Runnegar, 1983). Rats and mice injected with acutely toxic doses of M. aeruginosa cells or toxin extract die within 1 to 3 hr (Elleman, Falconer, Jackson and Runnegar, 1978). Liver damage has been noted

as early as 15 minutes and it has been suggested that liver damage is a direct effect of microcystin on the hepatocyte membrane (Aune and Berg, 1986). The immediate cause of mortality in acutely dosed animals is hemorrhagic shock (Runnegar and Falconer 1982; Ostensvik, Skulberg and Soli, 1981).

It has been reported in various studies that microcystin poses a health risk to humans (Falconer et al. 1983). The incidence of fatal human poisonings by blue green algal toxins is not known. There is evidence that contact irritations and gastroenteritis occur when swimmers come into contact with toxic blue green algae cells or water containing the released toxin (Skulberg et al. 1984). It is not known if the toxin(s) were absorbed percutaneously or via ingestion. There is increasing concern that toxic blooms might pose a health risk to people through 1) drinking contaminated water and 2) recreational water. So far, no study has been reported to determine if skin exposure to water contaminated with microcystin would result in the penetration of microcystin through human skin. Due to extreme toxicity, it is not possible to conduct in vivo studies in humans.

## EXPERIMENTAL

### Materials

Microcystin-LR was generously supplied by Dr. J.G.Pace, U.S. Army, Frederick, MD. Microcystin supplied was further purified by high pressure liquid chromatography to a purity of greater than 99 %. HPLC method followed was a little modification of the previously

described methods (Krishnamurthy et al, 1986; Dahlem, Hassan, Swanson et al, 1989). Reversed phase chromatography was performed with a unit from Waters Associates (Milford, MA), which consisted of a U6K Injector, Model 510 Pump, Model 490 Variable Wavelength Detector, Model 740 Data Module and Model 712 Waters Intelligent Sample Processor (WISP). Separation was performed on either a Waters uBondapak C18 steel column (10  $\mu$ m, 2 mm x 30 cm) or a Hypersil ODS column (5  $\mu$ m, 50 mm x 4.6 mm, Keystone Scientific, Inc., State College, PA). The eluant was 0.1 M ammonium acetate (Ph 4 to 6): acetonitrile (75:25, v/v) and was pumped at a flow rate of 0.5 ml/min. Absorbance was monitored at 240 nm. The microcystin peak had retention time of  $11 \pm 1$  min (mean  $\pm$  SD, Fig.2).

ICR female mice (n = 6) weighing  $27 \pm 0.5$  g (mean  $\pm$  SD) were used to test the toxicity and verify the identity of the microcystin peak. After injection of the microcystin into the HPLC, eluant was collected from 10 to 12 min with a fraction collector (ISCO Inc., Lincoln, Nebraska). The collected fraction was blown to dryness under a gentle nitrogen stream. The residue was dissolved in normal saline. ICR mice were dosed intraperitoneally (i.p.) with 20, 40 and 100  $\mu$ g/kg of microcystin in 20, 40 and 100  $\mu$ l of normal saline, respectively. For the control group, normal saline was injected into the HPLC and the eluant was collected at the same retention time as that of the microcystin peak (11 min). The collected fraction was evaporated to dryness under a nitrogen stream, re-suspended in normal saline and injected into the control

mice (n = 3). This was done to determine that toxicity of the HPLC eluant was not due to an HPLC contaminant, but was due to the microcystin eluting at 11 min. No effect was observed in the control group. The LD50 was determined to be 40 ug/kg, i.p. These results are consistent with the report by Eriksson, Meriluoto, Kujari and Skulberg, 1988.

The stability of microcystin in the receptor fluid was evaluated by mounting Teflon discs instead of skin, on two diffusion cells in each group. The receptor fluid was dosed with microcystin (5 ug/100 ul of water, 100 ug/25 ul of DMSO, 100 ug/50 ul of methanol). The cells with Teflon discs were incubated along with cells with skin discs, in an environmental chamber (37°C) for 48 hr. Each treatment group was replicated at least twice. Samples from receptor fluid were prepared and analyzed in the same manner as the receptor fluid samples collected from the diffusion cells with skin discs.

Mouse skin was obtained from 13 female ICR mice weighing  $30 \pm 2$  g (mean  $\pm$  SD). Guinea pig skin was obtained from 5 male Hartley guinea pigs, weighing  $550 \pm 10$  g (mean  $\pm$  SD). Mice and guinea pigs were purchased from Harlan Sprague Dawley Inc., Indianapolis, IN. Animals were killed using carbon dioxide and the ventral surfaces of the animals were carefully shaved with electric clippers before excising the skin. Human abdominal skin was obtained from autopsies (performed within 24 hr of death) of 3 males (ages 47 to 79 years) and 2 females (ages 64 and 70 years). Loose subcutaneous fat was removed from excised skin. Split thickness human skin (thickness

500 - 650  $\mu\text{m}$ ) was prepared with a dermatome [Padgett Dermatome, Division of Kansas City Assemblage Co (Kansas, MO)]. Full thickness mouse and guinea pig skin was used. The thickness of mouse skin was 600 to 700  $\mu\text{m}$  and that of guinea pig skin was 650 to 800  $\mu\text{m}$ .

#### Methods:

Discs of excised skin, each 2.8  $\text{cm}^2$  in diameter, were mounted on static teflon diffusion cells (Riley, 1983) consisting of an upper and lower chamber. The lower chamber (dermal side) had a volume of 2.6 ml and was filled with fluid (receptor fluid). The receptor fluid was phosphate buffered saline (PBS) containing 157 mg penicillin/litre, 250 mg streptomycin/litre and 250 mg amphotericin B/litre to reduce microbial growth. In some of the experiments, instead of PBS, Hanks Balanced Salt Solution (HBSS) with HEPES buffer and gentamicin 50 mg/litre was used as receptor fluid. HBSS was bubbled with 95% oxygen and 5% carbon dioxide to keep the skin discs viable (Collier, Sheikh, Sakr *et al.* 1988). The skin discs were placed horizontally between the two chambers so that the dermal side was bathed by the receptor fluid and the epidermal surface was exposed to ambient conditions in an environmental chamber for 48 hr. Temperature and relative humidity during the length of experiment was  $36 \pm 3^\circ\text{C}$  and  $29 \pm 5\%$  (mean  $\pm$  SD), respectively.

In all the experiments skin from each specimen was mounted on at least two diffusion cells in each group (3 to 6 cells/group) so that the variations in skin penetration were evenly distributed.

Each experiment was repeated at least three times except for the experiment with the vehicle methanol which was repeated one time. At time 0, the epidermal surfaces of mouse, guinea pig and human skin were dosed with 32 - 36  $\mu\text{g}/\text{cm}^2$  of microcystin dissolved in 2 ml of water, 25  $\mu\text{l}$  of DMSO, or 50  $\mu\text{l}$  of methanol. The epidermal surfaces were not occluded when methanol or DMSO was used as the vehicle but were occluded when water was the vehicle. This was done to simulate a condition of a person swimming or walking in water contaminated with blue green algae toxin.

To determine if constituents which might interfere with the HPLC determination of microcystin were leaching out of the skin, some of the skin discs were dosed only with vehicle, i.e., with 2 ml water, 25  $\mu\text{l}$  DMSO and 50  $\mu\text{l}$  methanol. Control cells holding the skin discs dosed with different vehicles were incubated (at 37°C) along with cells holding skin discs dosed with microcystin dissolved in different vehicles. At the end of the experiments, receptor fluid and skin discs dosed with vehicle only were analyzed in a similar manner as the skin discs dosed with microcystin dissolved in these different vehicles.

To determine the effect of dose of microcystin on penetration through guinea pig skin, epidermal surfaces were dosed with 3.6, 7.2 and 32  $\mu\text{g}/\text{cm}^2$  of microcystin dissolved in 10, 20 and 25  $\mu\text{l}$  of DMSO, respectively.

At the end of each experiment (i.e., after 48 hr of incubation), the surfaces of some of the skin discs were washed with soap and water to determine the amount of the dose remaining

on the skin surface. Each skin disc was then extracted twice with 10 ml acetonitrile. This procedure involved homogenizing the skin with a Brinkman homogenizer, centrifuging the homogenate and collecting the supernatant fluid which was then dried under a gentle stream of nitrogen. The residues were immediately dissolved in methanol. Prior to the injections into the HPLC, the receptor fluids from each experiment were washed on 3 ml C18 solid phase extraction columns (SPE, J.T. Baker, Phillipsburg, NJ). Recovery of microcystin from SPE columns was determined to be  $95 \pm 3$  percent (mean  $\pm$  SD). Each column was primed with 3 ml of methanol followed by 6 ml PBS or HBSS. Receptor fluid was then aspirated and microcystin eluted with 2 ml of methanol or acetonitrile. The eluant was evaporated to dryness under nitrogen stream and re-suspended in 200 ul methanol for injection into HPLC.

Total recovery of the dose applied to each skin disc was calculated by summing the amount recovered from skin extracts, amount penetrated into receptor fluid and amount recovered from dose left on the epidermal surfaces. The amount of dose recovered from skin wash was added to the amount left on the epidermal surfaces.

**Statistical analysis:** Percent of the dose that penetrated through the skin was analyzed by PC ANOVA (Human System Dynamics, Northridge, California), and significant mean differences were estimated by Newman-Keuls test.

## RESULTS AND DISCUSSION

### Penetration through human skin and effect of vehicle

Microcystin penetrates the excised human skin to a small extent. Over a 48 hr period, only  $0.9 \pm 0.3\%$ ,  $1.8 \pm 0.8\%$  and  $3.8 \pm 0.06\%$  (mean  $\pm$  SD) of the total dose applied penetrated through human skin when water, DMSO or methanol, respectively, was the vehicle (Table 1). Microcystin penetration through human skin did not differ significantly ( $P > 0.05$ ) when water or DMSO was the vehicle, penetration was significantly faster when methanol was the vehicle ( $P < 0.05$ ). At 48 hr, 15% and 18% of the dose was in the skin when water or DMSO was the vehicle, respectively, and was not significantly different from each other ( $P > 0.05$ ). The percent of the dose remaining on the epidermal surface was 80 and 75 % when water or DMSO, respectively, was the vehicle. The total dose recovered was greater when water was the vehicle (96%) than when DMSO or methanol was the vehicle (95 % with both) but was not significantly different from each other ( $P > 0.05$ ). DMSO is a dipolar aprotic solvent and is thought to displace bound water from skin, resulting in a looser skin structure (Scheuplein and Bronaugh, 1983). The penetrant presumably diffuses through the DMSO in the membrane. DMSO may alter the skin on contact, diffuse rapidly through it, increase skin permeability, and thereby promote the penetration of materials dissolved in it (Allenby, Crasey, Edginton and Shock, 1969). The structural alteration of epidermis induced by DMSO and its analogues is, to some degree, reversible

(Baker, H. 1968). The rationale for use of DMSO was 1) to enhance movement of toxin across the membrane of skin (Wood and Wood, 1975), which can then simulate the effects of abrasion on dermal penetrant, and 2) to determine the extent of penetration where laboratory personnel who are involved in extracting microcystin accidentally come in contact with microcystin in the presence of a penetration enhancer. The larger penetration of microcystin in vehicle methanol through excised cadaver human abdominal skin could be due to the damage caused to the skin. It has been documented that the low molecular weight, less viscous, and more volatile solvents like methanol are deleterious to the skin barrier (Scheuplein and Blank, 1971). It was important to determine the effect of methanol as a vehicle for the same reasons as described above for DMSO.

HPLC analysis of receptor fluid and skin extract from skin discs dosed with vehicle either water, DMSO or methanol showed solvent peaks only. This indicated that if constituents of skin leached out of skin into the receptor fluid, then these constituents either had longer retention times (> 20 min) or else they were not strong ultraviolet light (UV) absorbers.

Chromatographic analysis of receptor fluid and skin extracts from the human skin discs dosed with microcystin dissolved in different vehicles showed no peaks other than the solvent peaks and microcystin peak. Samples prepared from mouse or guinea pig skin dosed with microcystin in DMSO also showed only microcystin and solvent peaks. Seemingly, cutaneous metabolism of microcystin did

not occur in human, mouse or guinea pig skin when DMSO was the vehicle or in human skin when water and methanol were vehicles (Fig.3). Alternatively, metabolites of microcystin were not strong UV absorbers or perhaps they had longer retention times. Fairly good recovery of microcystin (96% of the dose applied) also suggested that cutaneous metabolism of microcystin did not occur in significant amount.

**Penetration through skin of different species:**

Penetration of microcystin through mouse, guinea pig and human skin was compared when DMSO was vehicle. Penetration through mouse skin was significantly greater ( $P < 0.05$ ) than through human and guinea pig skin (Table 2). Penetration through human skin was greater than through guinea pig skin, but was not statistically different ( $P > 0.05$ ). These results suggest that guinea pig skin is a better model of microcystin penetration through human skin when DMSO is the vehicle. Our findings are consistent with other reports that suggest that guinea pig skin is an appropriate model for human skin absorption (Anderson, Maibach, and Ango, 1980).

**Effect of different doses of microcystin on penetration:**

The effect of different doses of microcystin on penetration through guinea pig skin was studied. Guinea pig skin was chosen for this purpose because it was determined that this species is a good model of microcystin penetration through human skin. The percent of the microcystin dose that penetrated decreased as the dose was increased (Table 3). The penetration was not significantly different when the dose of microcystin was increased from 8.5 ug

to 17 ug ( $P > 0.05$ ) but was significantly less when the dose was increased to 88 ug ( $P < 0.05$ ). The decrease in the percent of the dose which penetrated at high doses could be due to saturation of the absorption process (Wester and Maibach, 1983). Increasing the dose decreased the percent of the dose that penetrated, but the total dose penetrated was increased.

#### CONCLUSION

In conclusion, guinea pig skin was a better model of microcystin penetration through human skin than mouse skin. This study indicated that microcystin penetrates excised skin relatively slowly when the vehicle is water, DMSO or methanol. These findings were consistent with a previous report that indicated a dermal dose of microcystin (5 - 10 times the LD50 by subcutaneous route) caused no lethality in guinea pigs (Wannemacher Jr., Bunner and Dinterman, 1988). Our findings suggest that limited cutaneous exposure to low doses of microcystin in water, DMSO or methanol probably poses little health risk.

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Table 1. Effect of vehicle on penetration of microcystin through human skin<sup>a</sup>.

Vehicle (Sample Size)	Amount Penetrated	Amount in Skin	Amount left over epidermal surface	Total Recovery
Water (6)	0.9 ± 0.3 <sup>b</sup>	12 ± 3.9	72.9 ± 8.8	92.1 ± 7.1
DMSO (10)	2.2 ± 0.8 <sup>b</sup>	12 ± 3.6	59.8 ± 6.9	75.8 ± 7.4
Methanol (4)	3.8 ± 0.06 <sup>c</sup>	----	67.6 ± 4.5 <sup>d</sup>	72.5 ± 4.3

<sup>a</sup>Values represent distribution of microcystin 48 hr after applying the dose on skin. Values are expressed as percent/dose (mean ± standard deviation).

<sup>b,c</sup>Values with different superscripts are significantly different ( $P \leq 0.05$ ).

<sup>d</sup>Includes amount in skin and left on epidermal surface.

Table 2. Penetration of microcystin through excised mouse, guinea pig and human skin disks (vehicle: DMSO)

Dose $\mu\text{g}/\text{cm}^2$	Species (n)	Percent dose penetrated
36	Mouse (7)	$3.2 \pm 1.3^a$
32	Guinea Pig (6)	$1.4 \pm 0.7^b$
36	Human (17)	$1.8 \pm 0.8^b$

<sup>a,b</sup>Values are means  $\pm$  standard deviation. Those with different superscripts differs significantly ( $P < 0.05$ ).

Table 3. Effect of different doses of microcystin on penetration through guinea pig skin (vehicle: DMSO)

Dose $\mu\text{g}/\text{cm}^2$ (n)	Total dose penetration ( $\mu\text{g}/\text{cm}^2$ )	Percent dose penetrated
3.2 (6)	$0.14 \pm 0.05$	$3.9 \pm 0.5^a$
7.2 (5)	$0.25 \pm 0.1$	$3.5 \pm 1.0^a$
32 (6)	$0.43 \pm 0.2$	$1.3 \pm 0.7^b$

<sup>a, b</sup>Values are means  $\pm$  standard deviation. Those with different superscripts differ significantly ( $P < 0.05$ )

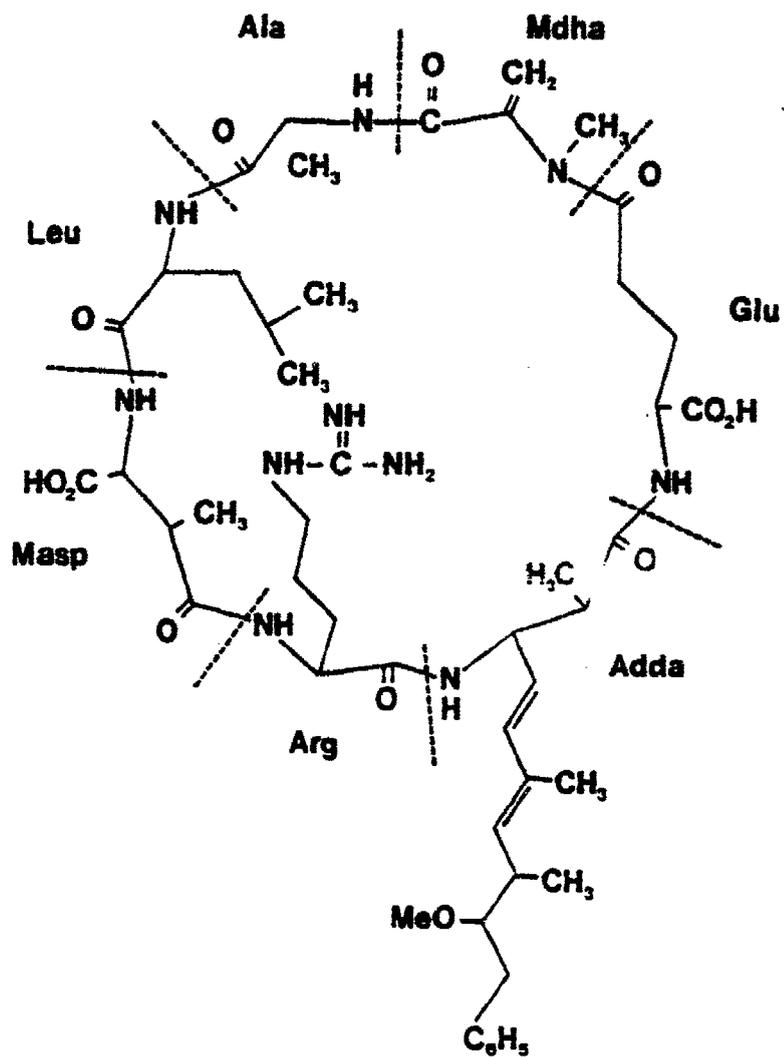


Figure 1. Structure of microcystin.

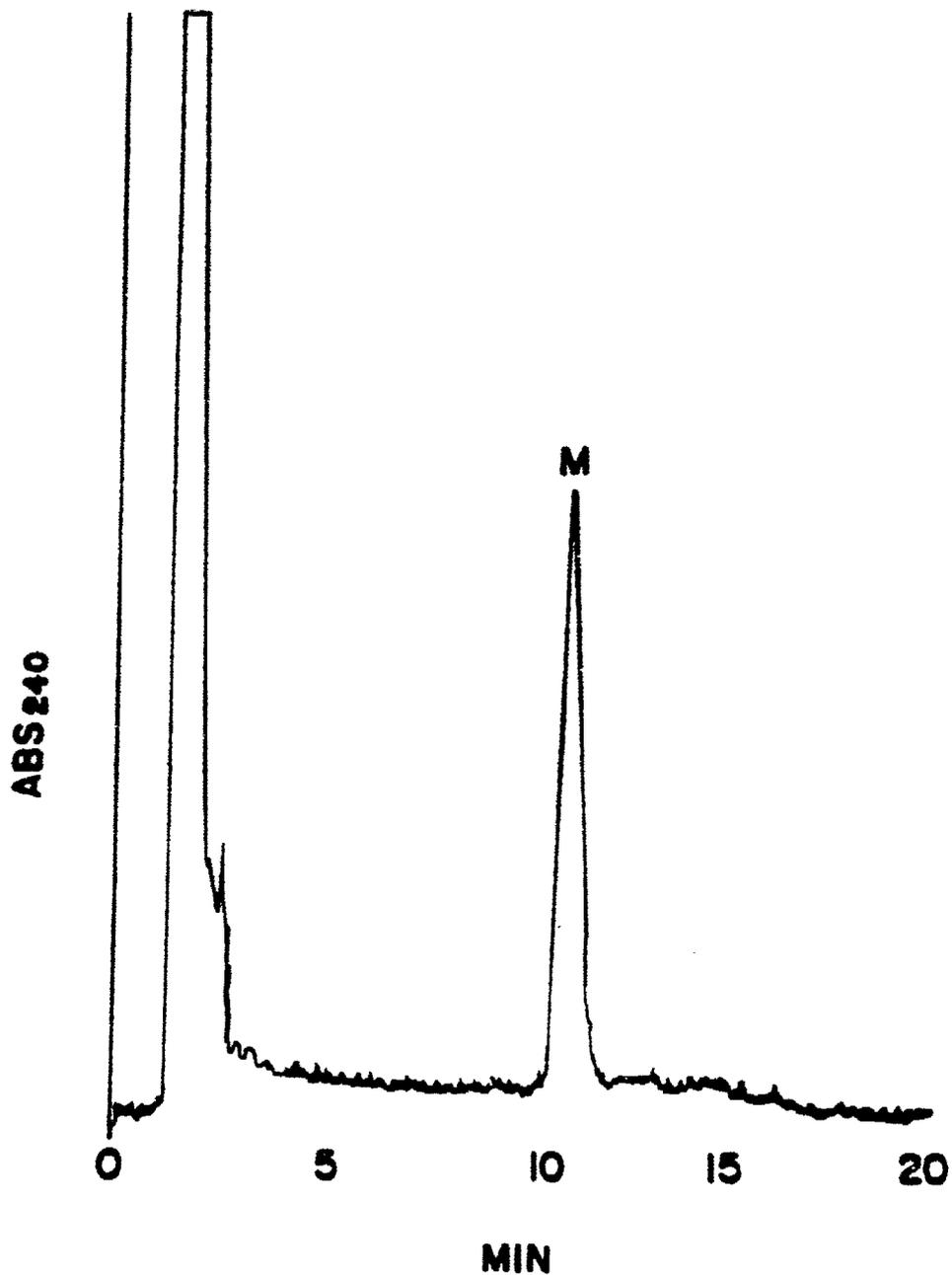


Figure 2. High performance liquid chromatography of purified microcystin (2.5 ug), M = microcystin peak. Chromatographic conditions were: reverse phase C18 uBondapak column, 10um, 3.9 mm x 36 cm; mobile phase - 0.1 M ammonium acetate (pH 4-6) : acetonitrile (75:25, v/v); flow rate - 0.5 ml/min; wavelength, 240 nm; AUFS, 0.1.

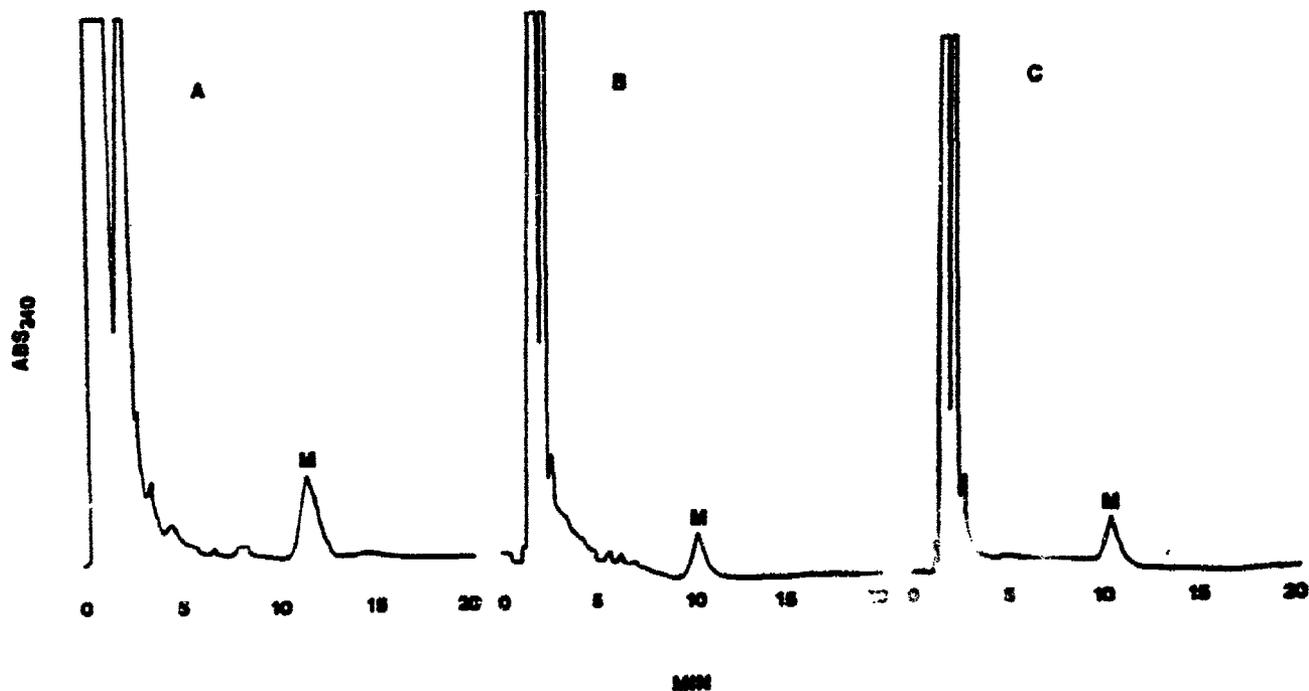


Figure 3. HPLC chromatographic analysis of receptor fluid from human skin dosed with microcystin dissolved in, A. 2 ml of water, B. 25 ul of DMSO and C. 50 ul of methanol. M= microcystin. Chromatographic conditions were same as described in figure 2.

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