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IN VITRO AND IN VIVO MEASUREMENT OF PERCUTANEOUS PENETRATION OF LOW MOLECULAR
WEIGHT TOXINS OF MILITARY INTEREST

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19. ABSTRACT

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SUMMARY

The purpose of these studies is to determine the rate of penetration of low molecular weight toxins through excised human skin and buccal mucosa. The compounds currently being studied are microcystin, brevetoxin ([³H]PbTx-3), lyngbyatoxin A, and saxitoxin. The methods involve mounting discs of excised skin on diffusion cells, applying the toxin to the epidermal surface, and measuring the accumulation of toxin in the receptor fluid which bathes the dermal surface of the skin disc. The diffusion cells are incubated at 36°C for the entire length of the experiment, 48 hr. High performance liquid chromatography (HPLC) has been used to measure the accumulation of microcystin and [³H]PbTx-3 in receptor fluid bathing excised skin. The penetration of microcystin through excised guinea pig, human and mouse skin was 1.5, 2.4 and 4.5 (expressed as percent of dose, 100 ug microcystin dissolved in DMSO). Analysis of the receptor fluid suggested that a fraction of the microcystin was metabolized during penetration through the human and mouse skin. When the dose of microcystin applied to excised guinea pig skin was decreased to 17 and 8.5 ug, there was an increase in the percent of dose which penetrated the skin (4.0 and 4.6%, respectively). When 0.9 ug of [³H]PbTx-3 (dissolved in DMSO) was applied to the epidermal surface of excised guinea pig skin, 12% of the applied dose penetrated the skin within 24 hr.



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TABLE OF CONTENTS

Summary	1
Foreword	11
List of Tables	v
List of Figures	vi
List of Investigators	viii
(1) Statement of Problem	1
(2) Background	1
Toxins being studied	1
Microcystin	1
Brevetoxin	2
Lyngbyatoxin A	2
Saxitoxin	3
Percutaneous penetration studies	3
(3) Rationale used in current study	5
(4) Experimental Methods	5
Source of toxins	5
Analytical methods	6
Microcystin	6
Brevetoxin	8
Lyngbyatoxin A	10
Saxitoxin	11
<u>In vitro</u> skin penetration methods	12
Microcystin	12
Brevetoxin	14
(5) Results and Discussion	15

(6) Conclusion	17
Literature Cited	19
Tables	22
Figures	29

LIST OF TABLES

Table 1.	Verify that HPLC peak (R_T = 6 to 7 min) is microcystin by determining lethality of peak (collected from HPLC eluate with fraction collector) in mice	22
Table 2.	<u>In vitro</u> penetration of topical microcystin through human, mouse and guinea pig skin (Vehicle:DMSO)	23
Table 3.	Analysis of microcystin and other peaks present in various components of percutaneous penetration system	24
Table 4.	<u>In vitro</u> penetration of topical microcystin in human skin (vehicle:methanol)	25
Table 5.	Effect of size of dose applied on penetration of microcystin through excised guinea pig skin	26
Table 6.	Analysis of brevetoxin ($[^3H]$ PbTx-3) and other radioactive peaks present in various components of percutaneous penetration system	27
Table 7.	Penetration of $[^3H]$ PbTx-3 through excised guinea pig skin (vehicle:DMSO)	28

LIST OF FIGURES

- Fig. 1. Chromatogram of microcystin standard. Chromatographic conditions included C₁₈ reverse phase column, mobile phase of 0.1 M ammonium acetate (ph 5.1):acetonitrile (75:25, v/v). Absorbance was monitored at 240 nm (same chromatographic conditions for figs. 6,7, and 8) 29
- Fig. 2. Chromatogram of brevetoxin (PbTx-2) standard. Chromatographic conditions were C₁₈ reverse phase column and mobile phase of methanol:water (85:15, v/v). Absorbance was monitored at 215 nm 30
- Fig. 3. Radiochromatogram of another brevetoxin ([³H]PbTx-3) standard. Chromatographic conditions were same as for Fig. 2. Radioactivity was monitored with a radioactive flow detector. The same radiochromatographic conditions were used for Figs. 9 and 10 31
- Fig. 4. Chromatogram of lyngbyatoxin A (teleocidin A) standard. Chromatographic conditions included C₁₈ normal phase column, mobile phase of hexane:chloroform:isopropanol (85:10:5, v/v), and flow rate of 0.5 ml/min. Absorbance was monitored at 254 nm 32
- Fig. 5. Diagram of static percutaneous diffusion cell used in percutaneous penetration studies 33
- Fig. 6. Chromatograms of receptor fluid bathing excised human skin dosed with a) 25 μ l DMSO; and b) 100 μ g microcystin in 25 μ l DMSO . . 34
- Fig. 7. Chromatograms of receptor fluid bathing excised mouse skin dosed with: c) 25 μ l DMSO; and d) 100 μ g microcystin in 25 μ l DMSO 35

- Fig. 8. Chromatograms of receptor fluid bathing excised guinea pig skin dosed with: a) 25 μ l DMSO and b) 100 μ g microcystin in 25 μ l DMSO 36
- Fig. 9. Radiochromatogram of receptor fluid bathing excised guinea pig skin dosed with 0.9 μ g [3 H]PbTx-3 37
- Fig. 10. Radiochromatogram of [3 H]PbTx-3 and other peak present in methanol extract of excised guinea pig skin, 48 hr after dosing epidermal surface with 0.9 μ g [3 H]PbTx-3 38

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1. STATEMENT OF PROBLEM

The purpose of this work was to determine (i) the amount of percutaneous absorption which would occur if humans were dermally exposed to low molecular weight (M.W.) toxins and (ii) the rate of penetration of low M.W. toxins through human mucosal membranes. Toxins which are currently being studied are microcystin, brevetoxin (PbTx-2 and PbTx-3), lyngbyatoxin A, and saxitoxin.

2. BACKGROUND

Toxins Being Studied. The following section provides a brief literature review of the toxins which are currently being studied in this contract.

Microcystin. Blue green algae poisoning causes disease and death of livestock and other animals that drink from algae infested waters. This group of algae, also known as cyanobacteria, is found world wide, but only a few of its members are poisonous. Known poisonous algae are microscopic and most of these are fresh water algae, though there are some poisonous marine forms also. Main genera responsible for fresh water toxic blooms are Microcystis, Anabaena, and Aphanizomenon. Most favorable conditions for a bloom to occur are warm, dry, low wind days of summer and early fall. Toxic blooms have been detected all over the world (1,2). Algae growth and toxin production is facilitated by increased environmental pollution and eutrophication of natural waters. Of the suspected toxic blue green algae, blooms of M. aeruginosa continue to be most commonly reported (3).

Microcystin (produced by strains of M. aeruginosa) is a cyclic peptide with M.W. of 994 daltons. Toxic bloom of blue green algae primarily represent a threat to wild life and domestic animals but there is increasing concern that they might also pose a health risk to people through: (i) drinking from contaminated water supplies and (ii) recreational water. People swimming in

algae contaminated waters have developed contact irritation and gastroenteritis (4). It is not known if the toxin(s) were absorbed percutaneously or via ingestion.

Brevetoxin. Brevetoxins are a group of highly lipid soluble compounds which are produced by the dinoflagellate Gymnodinium breve (renamed Ptychodiscus brevis). Dinoflagellate blooms, referred to as red tide, have been implicated in human intoxication resulting from ingestion of contaminated shellfish or exposure to seaspray aerosols. PbTx-2 (also referred to as T₃₄, brevetoxin B and GB-2) is primarily a neurotoxin and PbTx-3 (also referred to as T₁₇, dihydrobrevetoxin B and GB-3) causes bronchoconstriction (5,6). In mice the LD₅₀ of PbTx-3 is 94 ug/kg, via intravenous route. For humans there are several descriptions of signs and symptoms of exposure to brevetoxins. An aerosol of brevetoxins produced by shoreward wind lifting the foam from the water during a 'red tide' has caused coughing and sneezing (and possibly an asthmatic like attack) in persons and animal pets near the shore. Persons swimming in 'red tides' may experience eye and skin irritation and itching. Ingestion of shellfish contaminated with brevetoxins caused nausea, cramps, paresthesia of lips, face and extremities, occasionally weakness and difficulty in movement, even apparent paralysis, seizures and coma. Although severe illnesses have been reported, no deaths have been reported.

Lyngbyatoxin A (Teleocidin A). Lyngbyatoxin A is produced by the marine blue green algae Lyngbya majuscula. It has the same chemical structure as teleocidin A (10). Teleocidin A belongs to a class of teleocidins which also includes dihydroteleocidin B and teleocidin B. The teleocidin used in the present study is a mixture of 93% teleocidin A and 7% teleocidin B, and was isolated from Streptomyces mediterraneus as a strong skin irritant. Teleocidin belongs to a group of tumour promoters. Tumour promoters exert various biologic

effects in vivo and in vitro on cultured cells. These effects are thought to be induced after the tumour promoter bind to specific cell surface receptor (7,8). Teleocidin is as strong a tumour promoter on mouse skin as 12-O-tetradecanoylphorbol-13-acetate (TPA) (9,10).

Swimmer's itch was first reported in Japan in the summer of 1968 on Okinawa island. Symptoms were itching, rash, burning, blisters and deep erosion leaving a painful skin. Hashimoto (11) suggested that the marine blue green alga, L. majuscula, was the cause of this dermatitis in Okinawa. In the summer of 1980, there was a severe outbreak of swimmer's itch on Oahu Island, Hawaii, and aplysiatoxin and debromoaplysiatoxin from the marine blue green algae L. majuscula were identified as the causative agents (12,13,14). Later it was shown that these agents have potent tumor-promoting activity in 2 stage carcinogenesis in mouse skin (15,16,17).

Saxitoxin. Saxitoxin or paralytic shellfish poison is a nerve toxin, produced by Gonyaulax, a marine dinoflagellate (18). The toxin is a dibasic salt with pKa values of 8.2 and 11.5. The M.W. as a dihydrochloride salt is 354. As described above, favorable conditions lead to blooms of the dinoflagellates (algae) which are referred to as red tide. The toxins produced during blooms cause contamination of fish and shellfish (19). Human consumption of fish and shellfish contaminated with saxitoxin results in poisoning and occasionally death (20). The lethal dose is estimated to be approximately 1 mg in humans.

Percutaneous Penetration Studies. The low M.W. peptides and other compounds described above are all highly toxic. It is not known if dermal exposure to these compounds results in systemic effects. This study will determine to what extent these compounds penetrate skin and mucosal membranes. An in vitro technique for studying the rate of penetration of test material across skin has been developed which approximates, to varying degrees, the rate of penetration

which occurs in vivo (21,22,23). This study will also determine how accurately the in vitro penetration of low M.W. compounds predicts in vivo penetration by doing a series of in vitro studies which closely follow the conditions used in in vivo studies.

The effect of vehicle on the penetration of low M.W. toxins through intact skin is being evaluated. Polar solvents (for example, polyethylene glycol) are thought to enhance percutaneous penetration of a number of compounds through their role as solubilizing agents (24). Dimethylsulfoxide (DMSO) is a dipolar aprotic solvent which enhances the percutaneous penetration of many substances (25). DMSO is thought to act by displacing the bound water in skin and then the penetrant mainly diffuses through the DMSO in the membrane.

The penetration of low M.W. toxins through whole skin (epidermis and dermis), and buccal and nasal mucosa is being measured. The stratum corneum (the outer-most layer of the epidermis) is the main barrier to penetration through skin (26). The penetration of water through human dermis (epidermis removed after heating to 60°C) and dog buccal mucosa were 2 to 3 orders of magnitude greater than that of human epidermis (27). In man the stratified squamous epithelium of the mouth does not undergo complete keratinization (28). The absorption of some compounds via the buccal route is greater than by the oral route (29). For instance, buccal absorption of thyrotropin releasing hormone (a regulatory hormone consisting of three amino acids) results in a significant rise in thyrotropin and prolactin within 30 minutes (30). Insulin (M.W. = 6000) is readily absorbed via the intranasal route following administration of an insulin-bile salt aerosol (31). The nasal absorption of insulin is more similar to intravenous administration than subcutaneous or intramuscular (31). Preliminary studies indicate that intratracheal exposure to snake venom neurotoxins (M.W. = 9,000 - 40,000) results in toxicity. (32).

We have established that the in vitro system we are using is a good approximation of in vivo percutaneous absorption for [^{14}C]benzoic acid (which rapidly penetrates skin) and [^{14}C]urea (which is a slower penetrant). This was accomplished by measuring the penetration of [^{14}C] benzoic acid and [^{14}C]urea through excised human skin, and comparing our results with the results of in vivo absorption studies done in human with these compounds (21). In addition, we have shown that this same in vitro diffusion system provides a good approximation of in vivo skin absorption of [^3H]T-2 toxin (33).

3. RATIONAL USED IN CURRENT STUDY

The purpose of these studies is to determine the extent which these toxins penetrate through human skin. Humans are the species of interest but because of the extreme toxicity of these compounds, it is not feasible to measure their cutaneous absorption in human subjects. Consequently, penetration through excised human skin is being evaluated. In order to determine the relevance of the in vitro percutaneous penetration rates to in vivo absorption, we plan to compare in vitro and in vivo cutaneous penetration of the low M.W. toxins in guinea pigs or rats.

4. EXPERIMENTAL METHODS

Source of Toxins. Microcystin (LR form), in the powdered form, was generously supplied by Dr. J.G. Pace, U.S. Army, Frederick, MD. Microcystin was dissolved in methanol:water (10:90) and stored at -20°C . Microcystin (LA form) was generously supplied by Dr. H.W. Siegelman, Brookhaven National Laboratory, Upton, New York. The LA form was shipped dissolved in ammonium acetate and methanol, and was stored at -20°C . Brevetoxin (PbTx-2) was supplied by Dr. Mark Potl (U.S. Army, Frederick, MD). It was dissolved in 100% methanol and stored at

-20°C. [³H]PbTx-3 (purchased from Dr. D. Baden, University of Miami, Miami, FL) was dissolved in methanol and is currently being stored under nitrogen at -20°C. Lyngbyatoxin A (Teleocidin A) was generously supplied (in the powdered form) by Dr. Hirota Fujiki, Tokyo, Japan. It was dissolved in DMSO and stored at -20°C. [³H]saxitoxinol was generously supplied by Dr. J.G. Pace, U.S. Army, Frederick, MD. The [³H]saxitoxinol had a specific activity of 17.33 Ci/mmol. It was dissolved in methanol and stored at -20°C. Saxitoxin was generously supplied by Dr. S. Hall, FDA, Washington, D.C.

Analytical Methods

Microcystin (LR form). Purity of microcystin was determined by high performance liquid chromatography (HPLC) which was performed with a Waters system on a Waters uBondapak C¹⁸ reverse phase column (2mm x 30 cm). Eluent was ammonium acetate: acetonitrile (750:250, v/v) and flow rate was 0.5 ml/min. Absorbance was monitored at 240 nm, pressure was 2000 PSI. Initially 0.01 M ammonium acetate was used and pH was adjusted to 6.0. Retention time (R_T) for microcystin ranged from 6-9 minutes. The HPLC conditions were later modified to 0.1 M ammonium acetate and pH averaged 5.1 (it was not critical and varied from pH 4-6). With this latter mobile phase, the stock solution of microcystin was determined to contain 91% microcystin (R_T = 13 - 15 min), a contaminant which had a R_T of 11 min (3%), and another contaminant which had a R_T of 16 min (6%) (see figure 1). The HPLC was calibrated to determine linear relationship between amount (ug) of microcystin injected on HPLC and amount (area under curve, AUC) measured by HPLC. Amount injected was 0.05 ug to 5.0 ug of microcystin in 10 ul of methanol. Result was linear relationship between amount injected and area under the curve (correlation coefficient = 0.987). The lowest limit of sensitivity was 0.01 ug. External standard quantitation was used to calibrate integrator. Amount injected was 0.45 ug to 4.5 ug of microcystin and phosphate

buffered saline (PBSA) was the vehicle. The volume of each injection was 10 μ l. Linear relationship was obtained between amount injected (μ g) and concentration calculated by integrator (μ g) (correlation coefficient = 0.999). Same amounts of microcystin were injected with methanol or dimethylsulfoxide (DMSO) as the vehicle and calibration curve was accurate with these vehicles also.

Microcystin peak was verified by using a bioassay method. Microcystin peak was collected from HPLC eluate with a Retriever II (ISCO) fraction collector. It was evaporated to dryness under gentle nitrogen stream and dissolved in saline. An aliquot was injected onto HPLC to determine recovery of microcystin originally injected on HPLC. Approximately 50% of microcystin originally injected on HPLC was recovered in saline preparation. Chromatogram of saline preparation showed single peak, no contaminants were present. For control group, methanol was injected into HPLC, fraction was collected at the same R_f as that of microcystin. The eluate was evaporated to dryness under gentle nitrogen stream and was dissolved in saline. Female ICR mice were divided into 4 groups to determine LD₅₀ of microcystin in eluate. Each group contained 4 mice which were dosed intraperitoneally (IP). Group A was control, group B received 20 μ g/kg microcystin, group C received 40 μ g/kg microcystin, and group D received 100 μ g/kg microcystin. No effects were observed in the control group. Dose of 40 μ g/kg killed 50% of mice within 24 hr. Dose of 100 μ g/kg killed all the mice within 3 hr (Table 1).

A method was developed to prepare samples which contain microcystin. The method involves using solid phase extraction (SPE) columns to remove impurities and concentrate receptor fluid samples to be injected on the HPLC. PBSA is the receptor fluid which will bathe the dermal surface of the excised skin during in vitro penetration experiments. The method used was as follows:

- 10 aliquots of PBSA (volume of each was 2.5 ml) were spiked with 7.0 μ g of

microcystin by adding 10 μ l of stock solution (0.7 μ g of microcystin/ μ l 10% methanol)

- C₁₈ SPE columns (J.T. Baker) of 3 ml capacity were primed with 5 ml of methanol followed by 2.5 ml PBSA
- PBSA sample was pipetted onto column and was aspirated (using vacuum pump) until column was dry
- 1 ml of water was aspirated (to wash off the impurities)
- 1 ml methanol was aspirated, the eluate was dried down under nitrogen stream, dissolved in 100 μ l methanol, was injected (in duplicate) on HPLC to determine recovery.
- 3-4 samples were prepared on each column to determine if each column can be used more than once.

Results indicate that the recovery of microcystin (from PBSA samples eluted on SPE columns) increased with each sample which was run on a single SPE column. The mean recovery ($\pm$ S.D.) was $88 \pm 19\%$. In an attempt to decrease variability and increase recovery, sample preparation was modified. Now we use a single column for each sample. Column used is J.T. Baker with 3 ml capacity or Bond elut with 3 ml capacity.

Brevetoxin. Purity of brevetoxin (PbTx-2) was determined on a Waters HPLC. Waters μ Bondapak C₁₈ reverse phase column was used, mobile phase was 85% methanol: 15% water, with a flow rate of 0.5 ml/min. Absorbance was monitored at 215 nm. Chromatogram showed R_T of PbTx-2 to be 5.718 min and the purity was greater than 98% (see fig. 2). To determine sensitivity of HPLC system 5 μ l aliquots of dilutions of PbTx-2 in methanol were injected. Result was linear relationship (correlation coefficient = 0.999) between amount (μ g) of PbTx-2 injected and area under peaks within the range of 0.025 μ g and 2.5 μ g. R_T for PbTx-2 varied from 5.09 min to 5.72 min. Integrator was calibrated using Two-

Point External Standard Quantitation. Aliquots (5 μ l) of dilutions of PbTx-2 in PBSA (0.25 μ g, 0.5 μ g, 1.0 μ g, 2.5 μ g) were injected. Result was linear relationship between amount (μ g) PbTx-2 injected and concentration (μ g) calculated by integrator (correlation coefficient = 0.986). The accuracy of the calibrated integrator was determined for when methanol or DMSO was the vehicle for PbTx-2. Aliquots of PbTx-2 (dissolved in either methanol or DMSO) were injected. Result was linear relationship between amount injected (0.25 μ g to 1.0 μ g) and concentration (μ g) calculated by the integrator when methanol or DMSO was the vehicle.

The HPLC peak (R_T 5-6 min) for PbTx-2 was verified by using a bioassay. PbTx-2 peak was collected from HPLC eluate with a fraction collector (for details see method described above for microcystin). For control group, methanol was injected onto HPLC, eluate fraction (R_T = 5-6 min) was collected, evaporated to dryness and dissolved in 76 μ l of saline. Female ICR mice (2 per group) were dosed with IP injection of control preparation or PbTx-2 (200 μ g/kg). Dose of PbTx-2 was dissolved in 76 μ l of saline. No effects were observed in control group. The treated mice were depressed, not eating or drinking and had increased urination. After 24 hr one treated mouse died, while the surviving mouse appeared to have fully recovered. It was concluded that the HPLC peak, with a R_T of 5 to 6 min, was PbTx-2.

The purity of [3 H]PbTx-3 was determined with the same chromatographic conditions as described for PbTx-2 (see above). Radioactivity in the HPLC eluate was monitored with a radioactive flow detector (FLO-ONE, Radiomatic Instruments and Chemical Co, Inc, Tampa, FL). Scintillation counting solution used was FLO-SCINT II (Radiomatic Inc) and flow rate of counting solution was 1.5 ml/min. The stock solution of [3 H]PbTx-3 contained 85% [3 H]PbTx-3 (R_T = 6.1 min) and 15% contaminant (R_T = 3.7 min) (see Fig. 3). The [3 H]PbTx-3 sent by Dr. Baden had

contained 98% [^3H]PbTx-3. The rapid break down occurred because it had not been stored under nitrogen. One penetration experiment was done with the contaminated [^3H]PbTx-3 (see below) and the results indicated the contaminant penetrated excised skin faster than the [^3H]PbTx-3. Consequently, we found it necessary to use the HPLC to purify the [^3H]PbTx-3. The stock solution of [^3H]PbTx-3 is now 98% pure and is dissolved in methanol and stored under nitrogen at -20°C .

A sensitive and efficient method was developed for recovering small amounts of [^3H]PbTx-3 in receptor fluid. The method is as follows:

- SPE column used: 3 ml Reverse phase C18.
- Spiked 5 vials (each containing 2.5 ml of PBSA) with 5 μl of [^3H]PbTx-3 (49,976 DPM).
- Prime column with 5-6 ml of methanol.
- Aspirate 3 ml of PBSA.
- Apply sample to column, aspirate.
- Wash column with 2 ml of HPLC grade water.
- Elute with 2 ml of methanol, dry down eluate under gentle stream of nitrogen and low heat.
- Reconstitute with 200 μl of methanol, count 50 μl from each eluate on liquid scintillation counter.

Results indicated that the mean ($\pm$ S.D.) recovery of [^3H]PbTx-3 was $90 \pm 14\%$ (expressed as percent of amount spiked in 2.5 ml PBSA).

Lynngbyatoxin A. The purity of lynngbyatoxin A (teleocidin A) was determined with a Waters HPLC. A C₁₈ normal phase column (Partisil 5, Whatman) was used and eluent was hexane:chloroform:isopropanol (85:10:5, v/v), flow rate was 0.5 ml/min. Absorbance was monitored at 254 nm. Fig 4 shows a sample chromatogram. The stock solution was found to contain 98% lynngbyatoxin A and R_T was 9.547 min. To determine the lowest limit of HPLC sensitivity, various dilutions of

lyngbyatoxin A were made and aliquots of 5 μ l were injected on HPLC. Lowest limit of sensitivity was concluded to be 0.01 μ g using above mentioned column conditions. The integrator (Waters 740 Data Module) was calibrated by using External Standard Quantitation (two point calibration). Aliquots of lyngbyatoxin A (0.01 μ g to 0.5 μ g) in chloroform were injected on HPLC. A linear relationship between amount injected (μ g) and concentration calculated by integrator (μ g) was obtained (correlation coefficient = 0.999).

An effort was made to develop a sensitive and consistent method for measuring small amounts of lyngbyatoxin A in 2.5 ml volumes of PBSA (receptor fluid used in in vitro penetration experiments). The following method was used:

- Spike 5 vials (each containing 2.5 ml of PBSA) with 1 μ g of lyngbyatoxin A
- Prime normal phase SPE columns with 3-5 ml of hexane.
- Apply sample to the column, aspirate.
- Wash with hexane.
- Collect analyte by rinsing with 2 ml of chloroform.
- Dry down chloroform eluate, reconstitute with 100 μ l methanol, inject 10 μ l aliquots on HPLC.

Results indicated that the mean ($\pm$ S.D.) recovery of lyngbyatoxin A was 39 ± 34 % (expressed as percent of amount spiked in 2.5 ml PBSA). We plan to modify this method in order to increase recovery and reproducibility for measurement of lyngbyatoxin A.

Saxitoxin. We plan to use thin layer chromatography (TLC) and HPLC to determine the purity of the [3 H]saxitoxinol. Since [3 H]saxitoxinol has chemical characteristics similar to saxitoxin, published analytical methods for saxitoxin will be used (35,36). Saxitoxin will be used as a reference standard for [3 H]saxitoxinol because unlabeled saxitoxinol is not available. The relative mobility (R_f) of saxitoxin is less than the R_f of [3 H]saxitoxinol (F. Chu,

personal communication). The method for TLC involves spotting silica gel 60 TLC plates, developing the spotted plates in pyridine:ethyl acetate:acetic acid:water (15:5:3:4) for 3 hr. Saxitoxin will be visualized by spraying plate with 1% hydrogen peroxide, heating plate for 15 min at 120°C and checking fluorescence of spot at 365 nm. The [^3H]saxitoxinol will be measured with a Bioscan radiochromatogram scanner (Washington, D.C.). The HPLC methods involve an analytical cyano column (4.6 x 150 nm, Altex/Beckman). The mobile phase will be 10 mM $\text{NH}_4\text{H}_2\text{PO}_4$:methanol (50:40) with a flow rate of 1.0 ml/min. Absorbance will be monitored at 220 or 240 nm. The R_T for saxitoxin was reported to be 6.0 min (36). This method was reported to be sensitive to less than 2.9 ug saxitoxin. The TLC and HPLC methods described above will also be used to measure [^3H]saxitoxinol (and metabolites) in samples from the percutaneous penetration experiments.

In vitro skin penetration methods

Microcystin. The in vitro skin penetration by microcystin was evaluated in mouse, human and guinea pig skin. Skin was obtained from 13 ICR mice weighing 30 ± 2 gm ($\bar{x} \pm \text{S.D.}$) and from 2 guinea pigs weighing 541 ± 12 gm. Mice and guinea pigs were killed using CO_2 and ventral surface was carefully shaved before excising the skin. Whole thickness skin discs were prepared. Human abdominal skin was obtained from autopsies (performed within 24 hr of death) of 3 males (ages 47-79 yr). Subcutaneous fat was removed and split thickness skin was prepared with a dermatome (thickness was 500 μm). Excised skin discs were mounted on static teflon diffusion cells (figure 5). Dermal side of skin discs was bathed with PBSA (34) and epidermal surface was exposed to ambient conditions in environmental chamber for 48 hr. Mean temperature and relative humidity during experiments was 30°C and 36% ($n = 15$), respectively. Each experiment was repeated at least 2 times. At time zero, each epidermal surface was dosed with

25 ul DMSO or 100 ug microcystin dissolved in 25 ul DMSO. The large dose (100 ug microcystin) was used in order to facilitate HPLC measurement of microcystin in receptor fluid samples. At the end of each experiment each skin disc was homogenized with a Brinkman Polytron ultrasonic homogenizer (Switzerland) and extracted with methanol in order to determine the percentage of dose recovered. Microcystin and its metabolites in the skin extracts and receptor fluid were determined by HPLC. Receptor fluid samples were prepared with SPE columns before injecting into HPLC (method described above).

Stability of microcystin in receptor fluid (PBSA) was evaluated by mounting teflon discs, instead of skin, on 7 diffusion cells. Receptor fluid was dosed with 100 ug microcystin in 25 ul DMSO and incubated at 36°C for 48 hr. The stability of microcystin in DMSO was determined by dissolving microcystin in DMSO and measuring purity after incubation.

The effect of dose on in vitro penetration of microcystin through excised guinea pig skin was determined. Lower doses were used to be closer to the lethal dose for microcystin ($LD_{50} = 4 \text{ ug/kg}$ in mice, IP). Doses used were 8.5 ug/skin disc and 17 ug/skin disc. Vehicle was DMSO. Methods were same as described above.

We are currently determining the effect of vehicle on in vitro penetration of microcystin through human and guinea pig skin. The vehicles being studied are DMSO, methanol and water. The penetration of toxins through skin is assessed by measuring the accumulation of the toxin in the receptor fluid bathing the dermal side of the excised skin. The low solubility of microcystin (a highly lipophilic compound) in PBSA could inhibit diffusion of microcystin into the receptor fluid. Thus, microcystin might penetrate into the skin disc but not into the receptor fluid. This could result in artificially slow rate of penetration of microcystin through excised skin. This is not a problem when DMSO is the vehicle because

DMSO readily penetrates skin and acts as a strong solubilizing agent in the receptor fluid. However, when a small volume of methanol is the vehicle the methanol rapidly evaporates. Therefore the methanol does not penetrate the skin and does not act as a solubilizing agent in the receptor fluid. It has been shown that lipophilic receptor fluids can enhance the in vitro cutaneous absorption of lipophilic compounds so that there is improved correlation between in vitro and in vivo cutaneous absorption rates (38). The effect of receptor fluid on skin penetration by microcystin is being studied. The receptor fluids which are being compared are: (i) PBSA and (ii) 50% aqueous methanol. Excised human skin was dosed with 100 ug microcystin dissolved in 25 ul methanol. The stability of microcystin in each receptor fluid was evaluated by dosing receptor fluid (PBSA or 50% aqueous methanol) bathing teflon discs with 100 ug microcystin dissolved in 25 ul methanol. Cells were incubated in environmental chamber, for 48 hr, under ambient conditions of temperature (36°C) and relative humidity (36%). Other experimental methods were same as described above.

Brevetoxin. The in vitro penetration of [^3H]PbTx-3 through guinea pig skin was evaluated. Four discs of skin were excised from one guinea pig and mounted on static teflon diffusion cells. Teflon discs were mounted on two diffusion cells. The receptor fluid (PBSA) bathing the teflon discs was dosed with 0.9 ug [^3H]PbTx-3 in 106 ul DMSO. The epidermal surface of each guinea pig skin disc was dosed with 0.9 ug [^3H]PbTx-3 in 106 ul DMSO. The diffusion cells were incubated in an environmental chamber (37°C, 11% relative humidity) for 48 hr. The epidermal surfaces were non-occluded. The receptor fluid was sampled (50ul) at intervals of less than 10 hr, and the radioactivity in each sample analyzed with a Packard liquid scintillation counter. In addition, samples (25 or 50 ul) were removed from the receptor fluid after 24 and 48 hr of incubation, and the radiochemical composition of the samples was analyzed with the HPLC/FLC-ONE. At

the end of the experiment each disc of excised guinea pig skin was extracted twice with 10 ml methanol. In order to determine if it was necessary to homogenize the skin discs for efficient extraction, two of the skin discs were homogenized during extraction and two were intact. The radioactivity and radiochemical composition of the skin extracts was determined.

5. Results and Discussion

Microcystin. The relative amount of microcystin which penetrated through excised skin was guinea pig < human < mouse during 48 hr (Table 2). For each species the total penetration after 48 hr was less than 5% of the applied dose. The mean total recovery of the applied dose at the end of the experiment was 64% for the guinea pigs and greater than 85% for the mouse and human group. We are currently trying to increase total recovery by extracting skin with acetonitrile (instead of methanol) at the end of the experiments. The results of chromatographic analysis of receptor fluid is shown in Table 3. The stock solution of microcystin contained contaminants which had R_T of 11, 12 and 16 min. Microcystin was stable in DMSO and PBSA receptor fluid (for 48 hr) since the relative proportion of the contaminants remained constant in these groups. Analysis of receptor fluid (which had bathed the dermal surfaces of the excised skin discs dosed with microcystin) indicated a larger proportion of the contaminants and several new peaks (R_T = 9.5 min, 18.0 min, and 20.0 min). The larger proportion of contaminants in the receptor fluid indicate that the contaminants penetrate the skin faster than microcystin or that the skin increases the conversion (or metabolism) of microcystin into these other compounds. The presence of new peaks in the receptor fluid bathing the skin (but not in receptor fluid bathing teflon discs) suggests the skin is metabolizing the microcystin. These new peaks are not due to structural components of the skin

diffusing into the receptor fluid because these peaks were not present in receptor fluid bathing skin dosed only with DMSO (see Figs. 6,7 and 8).

The effect of vehicle (methanol) on the penetration of microcystin through excised human skin is shown in Table 4. We plan to discard these results because of the poor recovery (17% - 87%) of the applied dose at the end of the experiments. Subsequent to this experiment, we determined the reason for poor recovery (malfunctioning of demonstration model of auto injector on HPLC) and we are currently repeating this experiment.

The effect of size of dose on the penetration of microcystin through excised guinea pig skin is shown in Table 5. As the dose decreased (from 100 ug to 8.5 ug) there was a consistent increase in the percent of the dose which penetrated the skin. This is consistent with the finding that as the dermal dose of diacetoxyscirpenol (DAS) was decreased, there was an increase in the percent of dose which penetrated excised guinea pig skin (37). However, DAS penetrated excised guinea pig skin approximately 10 times faster than microcystin (37). Comparison of the penetration of DAS and microcystin through excised guinea pig skin must be done cautiously because of differences in the in vitro methods. In the DAS experiment the dose was 1.1 ug DAS dissolved in 100 ul DMSO and the diffusion cells were incubated at room temperature (23°C) for 48 hr. In the microcystin experiment the dose was 8.5 ug microcystin in 10 ul DMSO and the diffusion cells were incubated in an environmental chamber (36°C) for 48 hr.

The total penetration of microcystin through excised human skin was 2.35 (expressed as percentage of dose) during 48 hr of exposure. This value can not be compared to literature values for cutaneous penetration of microcystin because there are no published reports on cutaneous penetration of microcystin. The penetration of microcystin through excised human skin is generally small when compared to other compounds in the presence of DMSO. The total penetration of

T-2 toxin and verrucaric acid through excised human skin (during 48 hr of exposure was 29.2 and 10.3 (expressed as percent of dose), when DMSO was the vehicle (37). The penetration of hydrocortisone-4- C^{14} and hexopyrronium-methyl- C^{14} through excised human skin was 15.1 and 21.6 (expressed as percent dose penetrating per 24 hr) when DMSO was the vehicle (39). The *in vivo* cutaneous absorption of testosterone propionate- ^{14}C in humans was 34 (expressed as percent dose penetrated per 24 hr) when the vehicle was 90% alcoholic DMSO (40).

Brevetoxin. Table 6 shows the results of the radiochromatographic analysis of receptor fluid and skin extracts. There was some decomposition of $[^3H]PbTx-3$ in the PBSA receptor fluid since 85% of the radioactivity in the dose was associated with PbTx-3 and only 75% of the radioactivity in the receptor fluid bathing the teflon disc was associated with PbTx-3. The contaminant ($R_T=3.5$ min) present in the stock solution penetrated the skin faster than the PbTx-3. This is evidenced by the higher percent of the contaminant in the receptor fluid (31% at 24 hr, 36% at 48 hr) than in the skin extract (11%). Fig 9 and 10 show radiochromatograms of the receptor fluid bathing the excised guinea pig skin and an extract of the guinea pig skin, respectively. Because of the large proportion of contaminant, it was necessary to adjust the penetration data so that it represented ng of $[^3H]PbTx-3$. The penetration of $[^3H]PbTx-3$ through excised guinea pig skin was 12% of the dose after 24 hr and was still 12% of the dose after 48 hr (Table 7). This indicates that the diffusion of $[^3H]PbTx-3$ through the skin reaches equilibrium by 24 hr. At the end of the experiment 94% of the applied dose was recovered.

6. Conclusion

These preliminary findings indicate that both microcystin and brevetoxin (PbTx-3) penetrate excised skin. Microcystin appears to be metabolized by

excised skin. In vitro cutaneous metabolism of brevetoxin is minimal. Studies are currently being done to determine the effect of vehicle (DMSO, methanol, and water) on the penetration of microcystin and [^3H]PbTx-3 through excised human and guinea pig skin.

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Table 1. Verify that HPLC peak (R_T = 6 to 7 min) is microcystin by determining lethality of peak (collected from HPLC eluate with fraction collector) in mice.

Microcystin Dose ^a (ug/kg)	Lethality no. of mice dead (no. of mice dosed)
control ^b	0 (4)
20	0 (4)
40	2 (4)
100	4 (4)

^aFemale ICR outbred mice [Hsd:(ICR)BR; Harlan Sprague Dawley] weighing 23 to 28 gm, were dosed IP.

^bDose for control group was prepared by injecting methanol on HPLC and eluate fraction was collected which had the same R_T as microcystin. Eluate was evaporated to dryness and dissolved in saline.

Table 2. In vitro penetration of topical microcystin through human, mouse and guinea pig skin^a.

Species (n)	% of Applied Microcystin Recovered After 48 hr		Total Recovered
	Receptor Fluid ^b	Skin Extract ^c	
Human (6)	2.35 ± 1.08 ^d	86.7 ± 16.7	89.0 ± 16.9
Mouse (9)	4.45 ± 2.80	80.4 ± 17.1	85.0 ± 15.0
Guinea pig (2)	1.51 ± 0.76	58.4 ± 6.8	64.53 ± 12.0 ^e

^a100 ug of microcystin (LR form) dissolved in 25 ul DMSO was applied to epidermal surface of excised skin disc and incubated at 36°C for 48 hr.

^bMicrocystin in receptor fluid represents the total amount of microcystin which penetrated the excised skin during the 48 hr incubation period.

^cMicrocystin in the skin extract represents the amount of microcystin remaining on the skin surface or which had penetrated into (but not through) the layers of the skin.

^dValues expressed as mean ± standard deviation.

^eLow recovery in guinea pig group could be due to poor extraction of microcystin from homogenized skin with methanol. This experiment will be repeated and skin will be extracted with acetonitrile.

Table 3. Analysis Of Microcystin And Other Peaks Present
In Various Components Of The Percutaneous Penetration System

Material analyzed (n)	Microcystin and other peaks ^a						
	1 R _T ^b	2	3	Microcystin 13.2	4	5	6
	9.5	10.9	12.1		16.1	18.0	20.0
Stock solution (3)	0	2.4±0.2 ^c	0.7±0.1	90±1.6	6.2±0.7	0	0
Dose applied in DMSO (1)	0	2	1	89	7	1	0
Receptor fluid from teflon cell (5)	0	3.5±1.0	1.4±0.9	83.5±1.5	6.6±0.5	0	0
Receptor fluid from human skin (6)	9.5±14.6	4.8±4.5	5.0±5.2	58.9±20.3	16.1±8.7	1.9±2.5	3.8±5.4
Receptor fluid from mouse skin (7)	0	12±10	7.4±7.9	64±14	9.9±4.8	6.3±6.5	0
Receptor fluid from guinea pig skin (8)	0	17.9±12.5	0	75.5±12.4	6.6±6.6	0	0

^aExpressed as relative percent peak area

^bR_T = Retention Time (min).

^cMean ± standard deviation.

Table 4. In vitro penetration of topical microcystin in human skin (vehicle:methanol).

Receptor fluid	Barrier	% of Applied Microcystin Recovered After 48 hr		
		Receptor fluid	Skin Extracts	Total Recovery
PBSA	Teflon disc	87.2±9.3 ^a		87.2±9.3
	Human skin	3.9±0.1	34.8±2.2	38.6*
PBSA:MECH 50:50	Teflon disc	17±1.4		17±1.4*
	Human skin	4.2±0.5	40.0±0.8	44.1*

^aMean ± S.D.

*Low recovery of dose was probably due to malfunctioning WISP (auto injector for Waters HPLC). This experiment will be repeated.

Table 5. Effect of size of dose applied on penetration of microcystin through excised guinea pig skin^a.

Dose (ug microcystin)	n	% Applied Microcystin Recovered After 48 hr		
		Receptor fluid	Skin extracts	Total recovery
8.5	4	4.63±0.93 ^b	62.25±7.1	66.75±8.8
17	4	3.95±0.94	45.0 ±2.9	49.5 ±3.3
100	8	1.31±0.67	8.83±7.7	60.13±8.0

^aDiscs of excised guinea pig skin were mounted on static diffusion cells. The epidermal surfaces were dosed with 25 ul DMSO (controls) or microcystin dissolved in 10-25 ul DMSO. The epidermal surfaces were non-occluded and the diffusion cells were incubated at 36°C for 48 hr.

^bExpressed as mean ± standard deviation.

Table 6. Analysis of brevetoxin ($[^3\text{H}]\text{PbTx-3}$) and other radioactive peaks present in various components of percutaneous system.¹

Material Analyzed	Chromatographic peaks ²	
	Contaminant	$[^3\text{H}]\text{PbTx-3}$
	$R_T = 2.8-4.1$	5.6 - 7.1
$[^3\text{H}]\text{PbTx-3}$ Stock Solution (dissolved in Methanol)	14 ^a	86 ^a
$[^3\text{H}]\text{PbTx-3}$ applied to skin (dissolved in DMSO)	15 ^a	85 ^a
Receptor fluid bathing teflon ^R disc (48 hr after dosing)	24, 24 ^b	75, 75 ^b
Receptor fluid bathing guinea pig skin		
24 hr after dosing	31 $\pm$ 2 ^c	69 $\pm$ 2 ^c
48 hr after dosing	36 $\pm$ 1 ^c	64 $\pm$ 1 ^c
Extract of guinea pig skin		
1st extract	9 $\pm$ 1 ^c	89 $\pm$ 2 ^c
2nd extract	8 $\pm$ 1	78 $\pm$ 2

¹Discs of excised guinea pig skin were mounted on static diffusion cells. The epidermal surfaces were each dosed with 0.9 μg $[^3\text{H}]\text{PbTx-3}$ dissolved in 106 μl DMSO. Samples (25 to 50 μl) were removed from the receptor fluid (24 and 48 hr after dosing) and radiochemical purity analyzed with HPLC/FLO-ONE. The stability of $[^3\text{H}]\text{PbTx-3}$ in receptor fluid was determined by incubating $[^3\text{H}]\text{PbTx-3}$ in PBSA bathing teflon discs for 48 hr.

²Expressed as percentage of total peak area. R_T = retention time (min).

^aResult of single determination.

^bIndividual data points listed (n=2).

^cMean $\pm$ standard error (n=4).

Table 7. Penetration of [^3H]PbTx-3 through excised guinea pig skin (vehicle:DMSO)

Components of Percutaneous Diffusion System	[^3H]PbTx-3 ^a	
	ug	% of applied dose
Dose applied to epidermal surface of excised guinea pig skin	0.933	
Dose present in receptor fluid		
- after 24 hr incubation	0.110 $\pm$ 0.012	12
- after 48 hr incubation	0.109 $\pm$ 0.012	12
Dose recovered in methanol extracts of skin		
- first extract	0.716 $\pm$ 0.018	76
- second extract	0.060 $\pm$ 0.015	6
Total recovery of applied dose	0.885	94

^aAll values have been adjusted to represent [^3H]PbTx-3, based on results of radiochromatographic (HPLC) analyses of dose applied, receptor fluid and skin extracts (See Table 6)

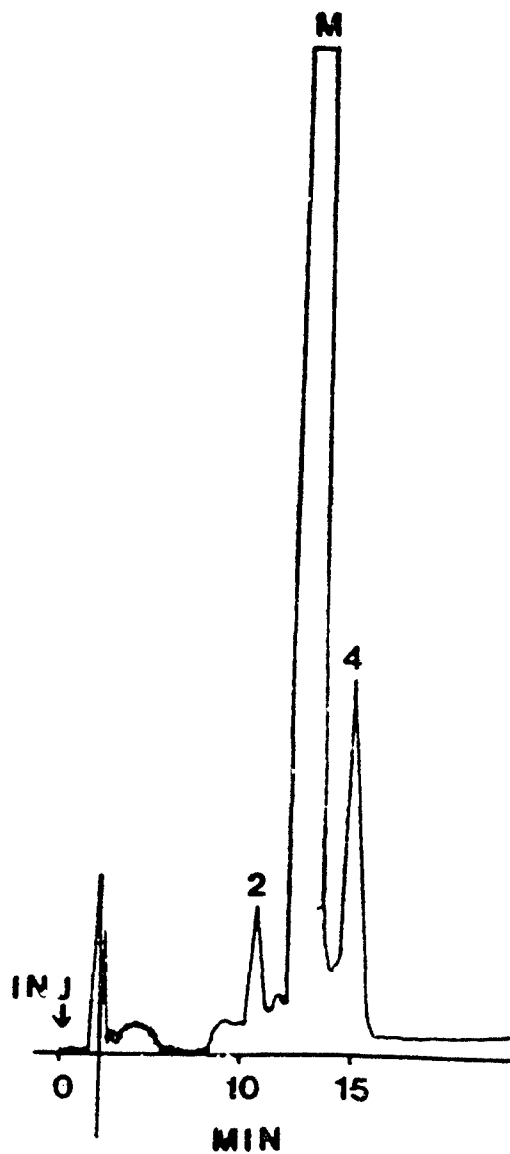


Fig. 1. Chromatogram of microcystin standard. Peaks: 2=contaminant; M=microcystin (LR form), 5 ug; 4=contaminant. Chromatographic conditions: u Bondapak C18 reverse phase column, mobile phrase was 0.1 M ammonium acetate (pH 5.1): acetonitrile (75:25,v/v), absorbance was monitored at 240 nm.

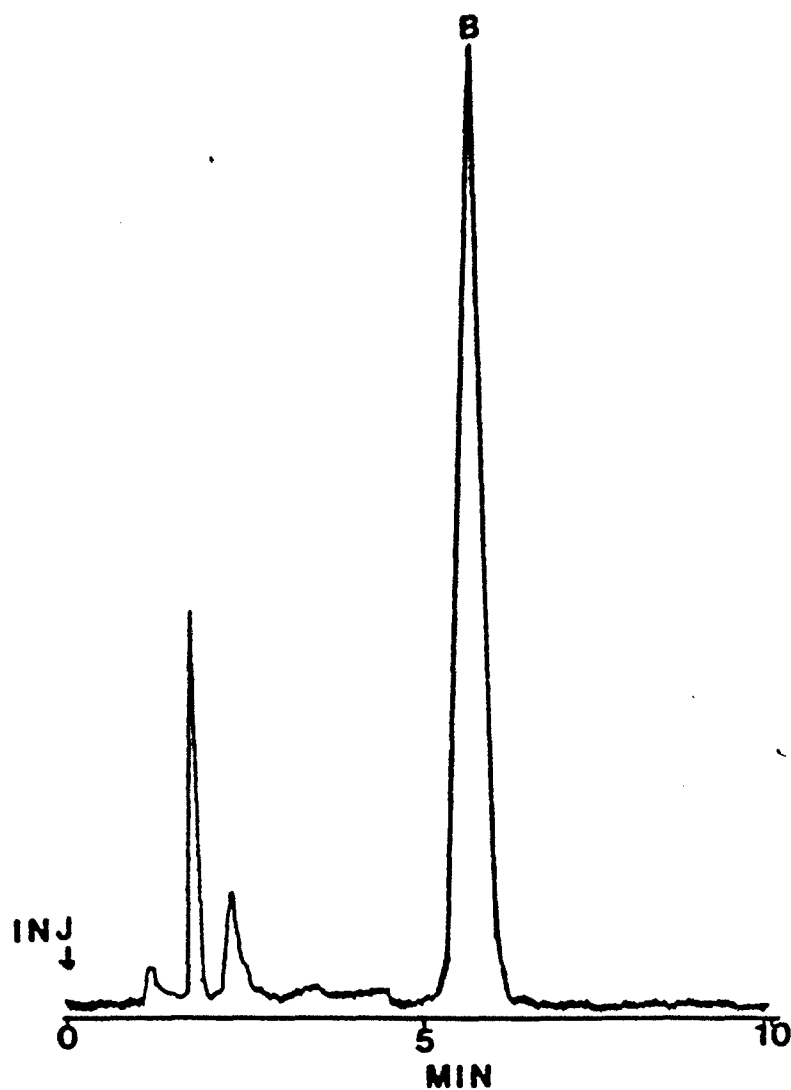


Fig. 2. Chromatogram of brevetoxin (PbTx-2) standard. Peak: B=PbTx-2, 5 ug. Chromatographic conditions: μ Bondapak C18 reverse phase column, mobile phase was methanol: water (85:15, v/v), absorbance was monitored at 215 nm.

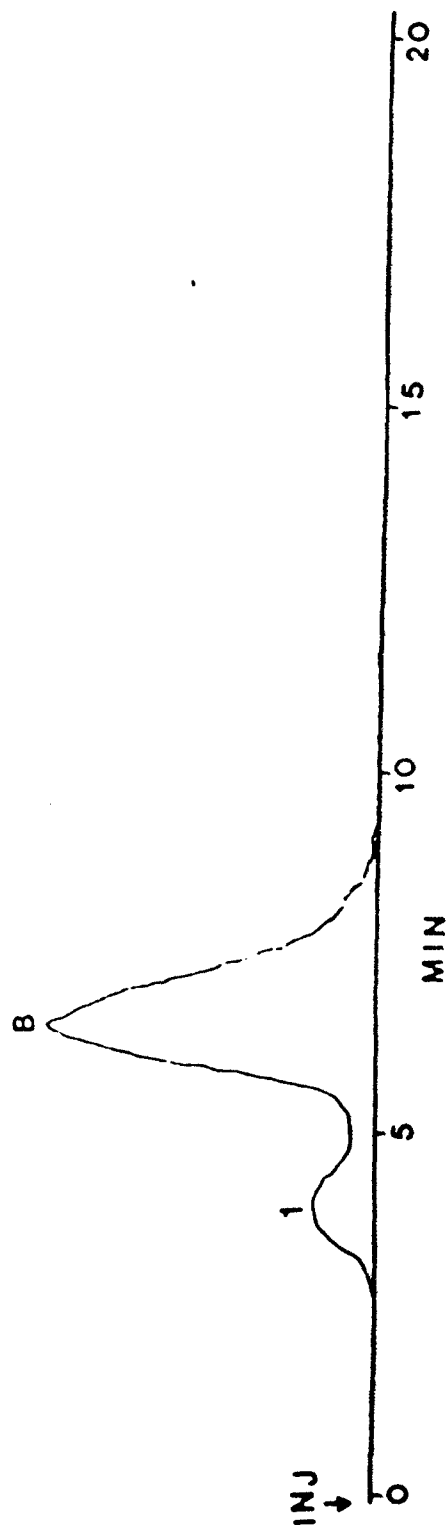


Fig. 3. Radiochromatogram of [^3H]PbTx-3 standard. Peaks: B = [^3H]PbTx-3, 2.3×10^{-2} μCi ; 1 = [^3H] contaminant. Chromatographic conditions: u Bondapak C₁₈ reverse phase column, mobile phase was methanol: water (85:15, v/v), radioactivity monitored with FLO-QNER. The same chromatographic conditions were used for Figs. 9 and 10.

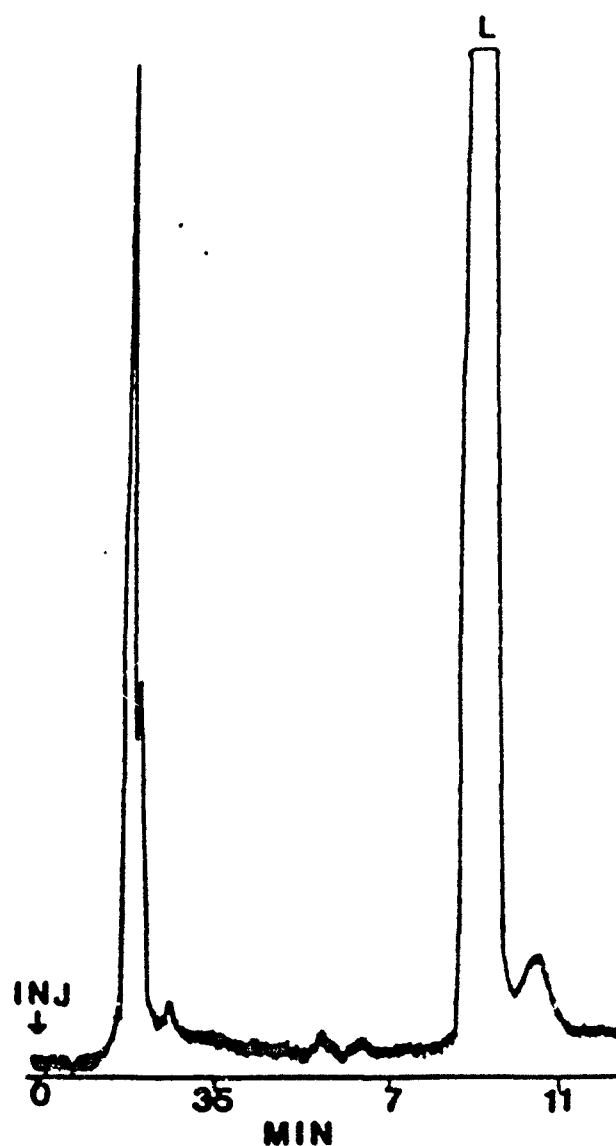


Fig. 4. Chromatogram of lyngbyatoxin A standard. Peak: L = lyngbyatoxin A, 5 ug. Chromatographic conditions: C18 normal phase column, mobile phase was hexane: chloroform: isopropanol (85:10:5, v/v), and absorbance was monitored at 254 nm.

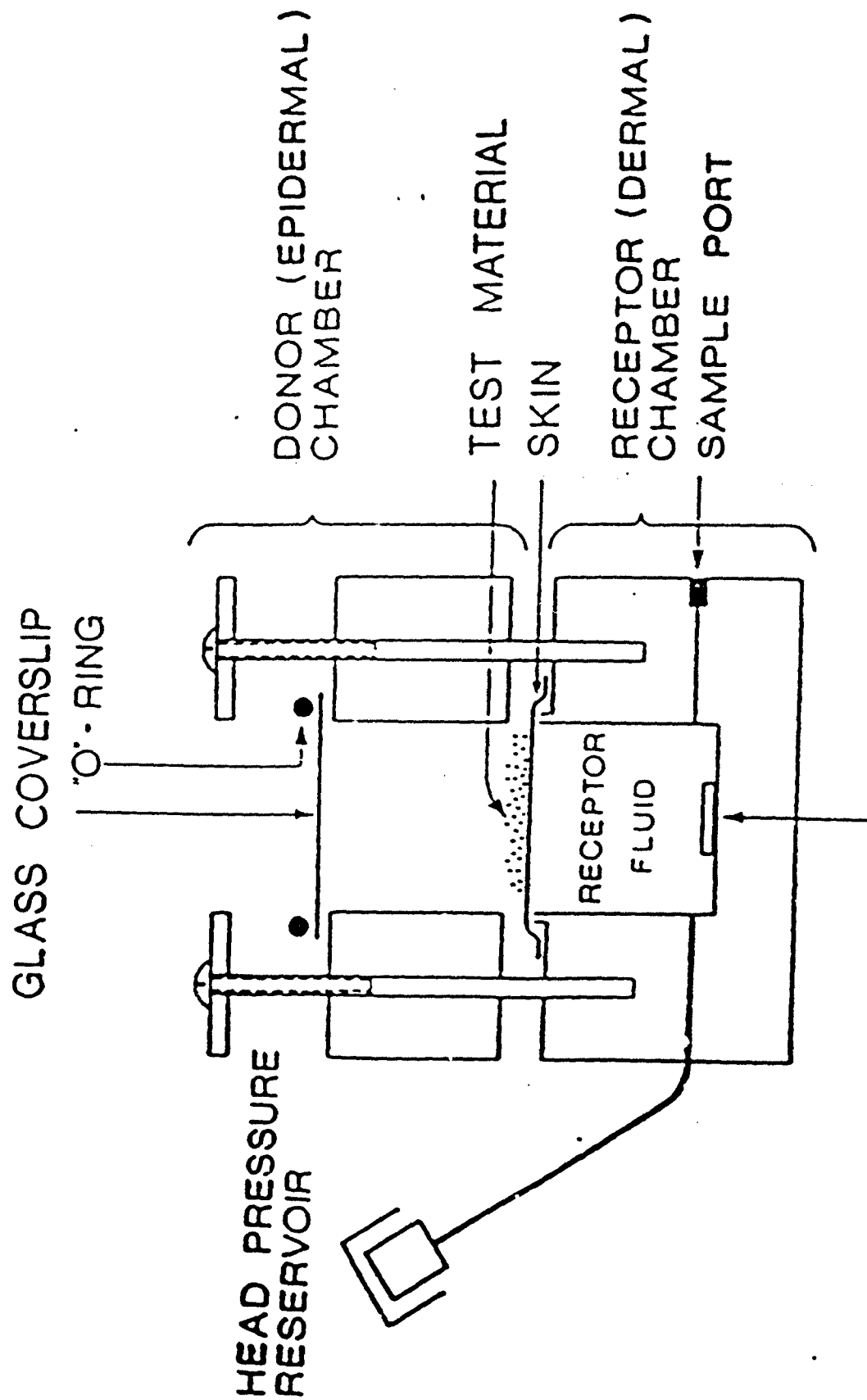


Fig. 5. Diagram of static diffusion cell used in percutaneous penetration studies. Glass coverslip was not used in these studies.

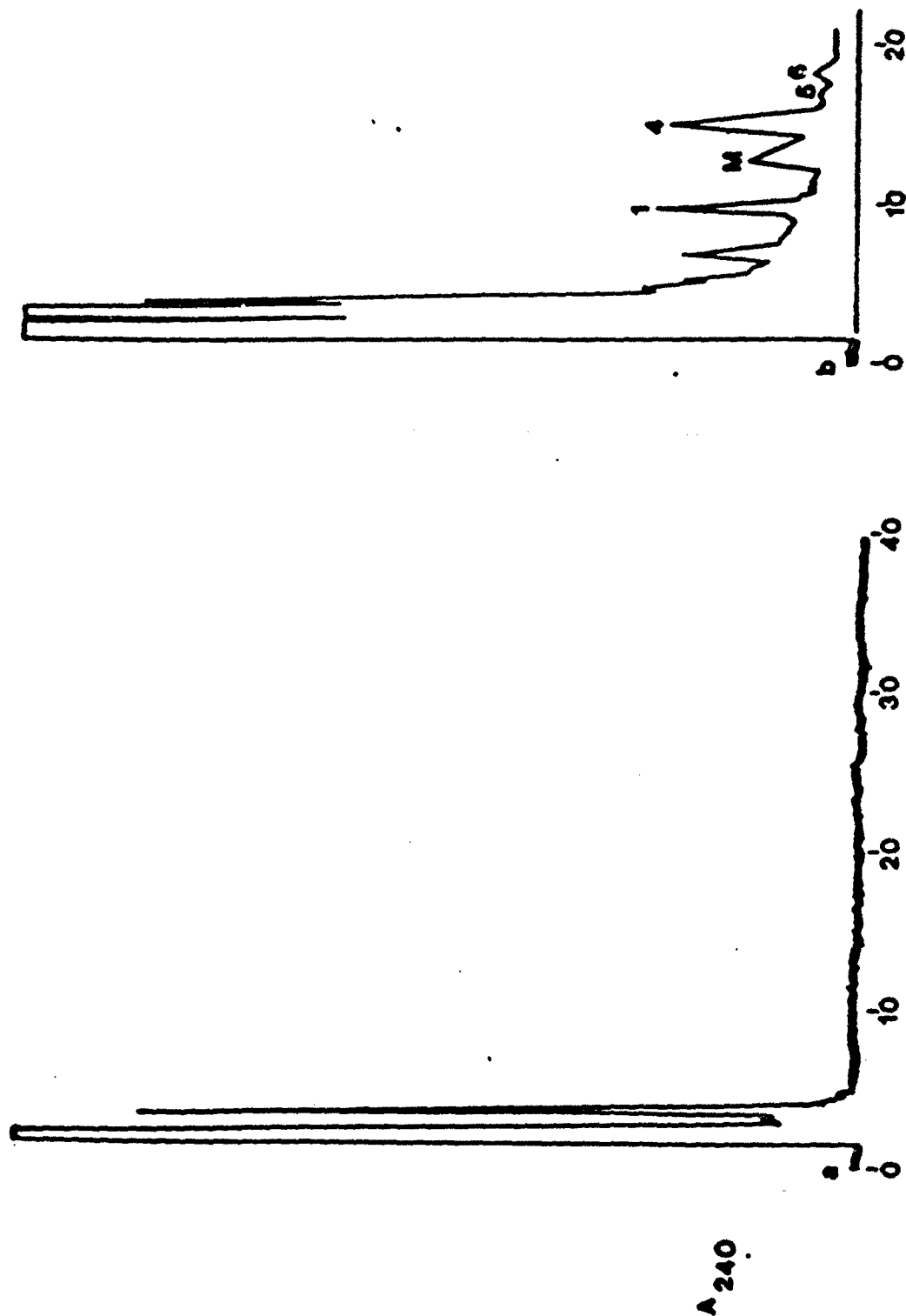


Fig. 6. HPLC chromatograms of receptor fluid bathing excised human skin dosed with:
a) 25 μ l DMSO; and b) 100 μ g microcystin in 25 μ l DMSO. Peak 4 is a contaminant
present in stock solution of microcystin; Peaks 1, 5 and 6 are metabolites of
microcystin: Peak M is microcystin.

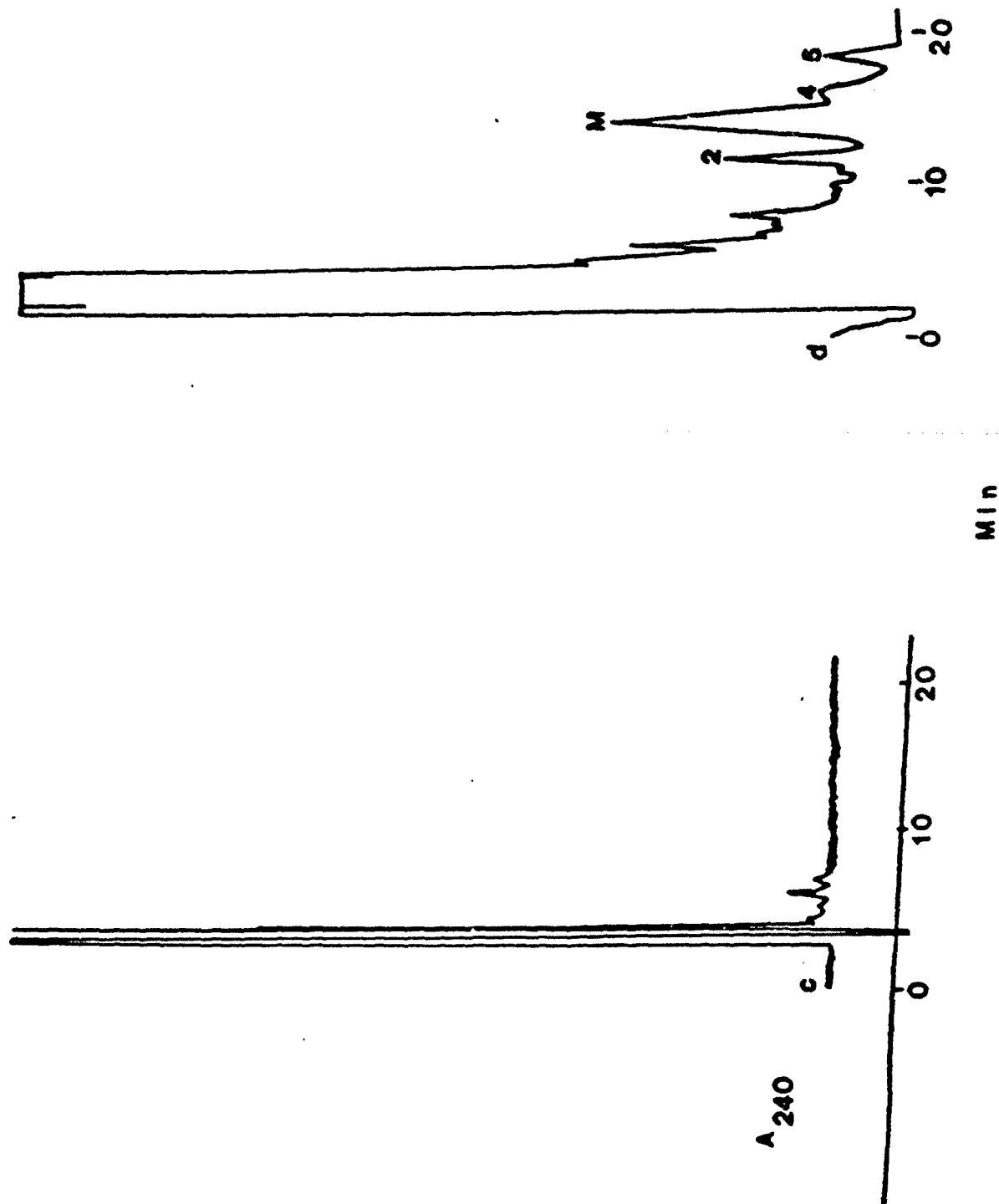


Fig. 7. HPLC chromatograms of receptor fluid bathing excised mouse skin dosed with: c) 25 μ l DMSO; and d) 100 μ g microcystin in 25 μ l DMSO. Peaks 2 and 4 are contaminants present in stock solution of microcystin; Peak 5 is a metabolite of microcystin; and Peak M is microcystin.

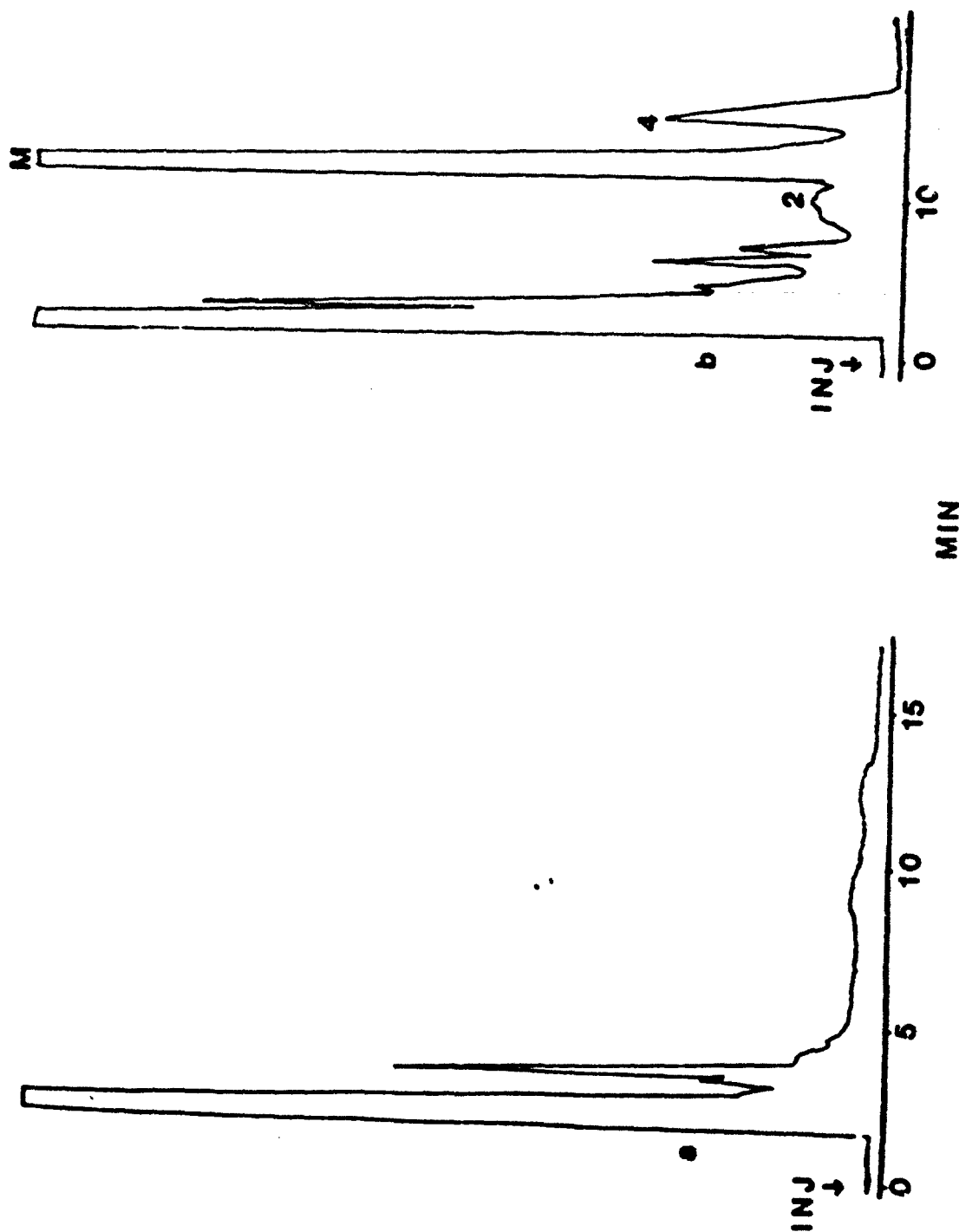


Fig. 8. HPLC chromatograms of receptor fluid bathing excised guinea pig skin dosed with: a) 25 ul DMSO and b) 100 ug microcystin in 25 ul DMSO. Peaks 2 and 4 are contaminants present in the stock solution of Microcystin; peak M is microcystin.

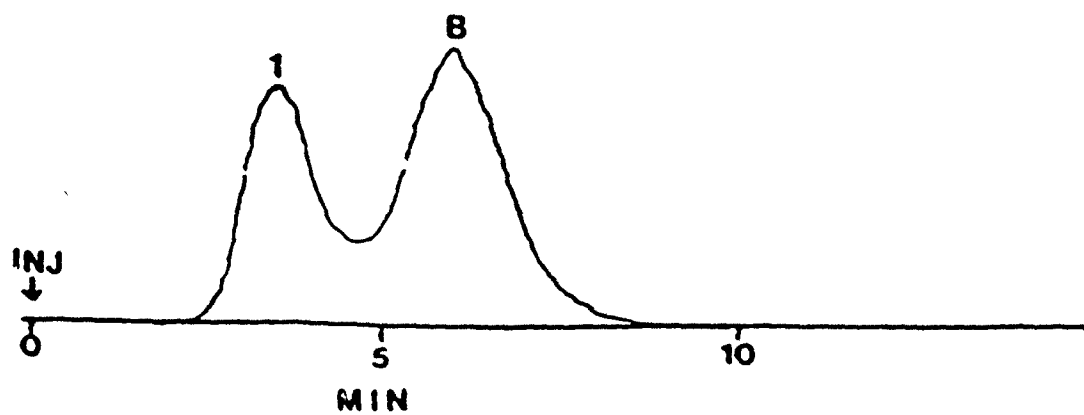


Fig. 9. Radiochromatogram of receptor fluid bathing excised guinea pig skin dosed with 0.9 ug [^3H]PbTx-3. Peak 1 is a contaminant present in the stock solution of [^3H]PbTx-3, peak B is [^3H]PbTx-3.

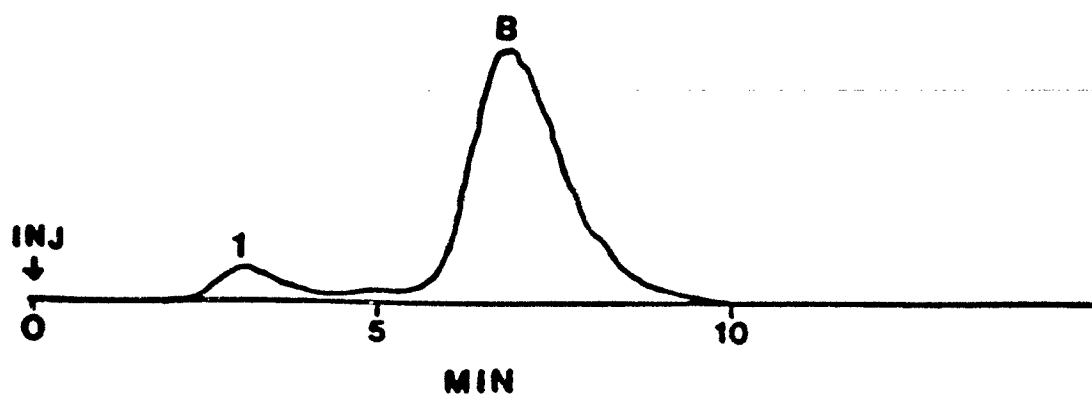


Fig. 10. Radiochromatogram of [^3H]PbTx-3 and other peak present in methanol extract of excised guinea pig skin, 48 hr after dosing epidermal surface with 0.9 ug [^3H]PbTx-3.

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