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ETHNOPHARMACOLOGIC RESEARCH: DISCOVERING NEW PROTOTYPE DRUGS THAT WILL BE USEFUL IN THE CLINIC.

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INTRODUCTION

Drug therapy still remains the cornerstone of medical treatment in general and clinical practice in particular. Drug development in the western world has become a very expensive business requiring the outlay of billions of dollars to develop a single drug for therapy. The cost and limitations of new avenues for exploration has significantly slowed the development of new pharmacological agents that are both effective and selective for specific indications. Added to these obstacles are the bereaucratic impediments typified by the stringent Food and Drug Administration requirements before even a desirable agent can be approved.

African Medicine Man (AMM) as distinguished from the Magician, provides one of the least exploited source for breaking through the confines which have limited the search for new pharmacologic agents with therapeutic benefits. In our judgement, the African folk medicine, which the AMM practises provides new hope and frontier for development of new drugs to be used for the benefit of mankind. Here is a new fertile, unexplored research area which is as broad and expansive as the mind could envision. Several questions come to mind: How could one exploit this area? What is the best approach? Specifically, could one select a particular medicine of known origin and identify the pharmacologically and toxicologically active component(s)?

One of the major interest of our laboratory is pharmacodynamics with special emphasis on muscle pharmacology. Therefore, we decided

to break into this virgin territory of African folk medicine by undertaking an extensive toxicological and pharmacological studies of an African arrow poison of plant origin which had been observed to kill the prey through muscle paralysis.

1. Acute toxicity studies of the crude toxin to provide basic toxicological and pharmacological information necessary for identifying the isolated active component (s) at a later stage;
2. Separation of the active components of the crude toxin using chromatographic (Thin Layer, Ion-exchange and High Performance Liquid Chromatography) techniques;
3. Quantification of the crude and isolated components of the toxin using physical (High Performance Liquid Chromatography) and bioassay methods;
4. Structure determination of the separated components using spectrometric (Mass, Infra Red, Ultraviolet and Nunclear Magnetic Resonance) techniques and;
5. Pharmacological and toxicological studies of each of the isolated components to elucidate the mechanism of action and establish safety.

The potential of identifying and characterizing the active component(s) of the arrow poison is great. For example, the muscle relaxing component would be useful in surgical operation, electroconvulsive therapy, and also in other therapeutic modalities requiring muscle relaxation.

The purpose of this presentation is to present the data which

we have put together from the investigative efforts on the toxin funded by the A.I.D. small grant awarded to me for the research.

MATERIALS AND METHODS

1. Arrow Poison

Arrow poison was heat-extracted from plants by the natives of Kenya from where the sample used in this investigation was obtained. The procudure for preparation and the types of plants from which the toxin is extracted are in the process of being classified botanically and will be reported elsewhere. The poison was a dry extract which easily dissolved in water and/or physiological saline.

2. Acute Toxicity Studies

a). Determination of Lethality

The arrow poison was dissolved in physiological saline solution. The lethality of the poison after injection into the mouse tail vein was computed according to the method of Litchfield and Wilcoxon (1949). Male Sprague-Dawley mice weighing 15-22g were used in the studies. Any peripheral effects of the posion on the mouse after injection were noted until death. The median toxicity (LD50) was obtained from semilogarithmic plots of the data, and the limits at the 95% confidence level was converted to SEM by the method of Miller and Tainter (1944).

b). The Effect of Cholinergic Antagonists on the Lethality of Arrow Poison.

Mice were divided into 4 groups. The first group was used as a control. The second, third and fourth groups were each pretreated (i.p) with atropine (10 mg/kg), physostigmine (.25 mg/kg), and d-tubocurarine (2 mg/kg) fifteen minutes prior to the determination

of lethality of arrow poison in these groups of mice. The selected dose for each antagonist was the dose which we had established to elicit the desired blockade or stimulation. The LD50 for each group was then determined as described above.

c). The Effects of Sex on the Lethality of Arrow Poison

Sprague-Dawley mice weighing 15-20g were separated into two groups: male and female. The lethality of the arrow poison in each group was determined as outlined above. Any peripheral effects of the poison in each group were noted for comparative purposes.

d). The Effects of Arrow Poison on Respiration

Sprague-Dawley rats weighing between 300-400g were used. The rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p). The respiration rate was measured using a pair of subcutaneous needle electrodes, one of which was placed on each side of the chest. The electrodes were connected to an impedance pneumograph coupler (Type 7212, Narco Biosystems, Houston, TX). The setting of the coupler and amplifier was as follows: filter, 3; variable scale 20 mV/cm; and the polarity was +. The respiratory rate just before giving the poison was taken as the control rate and any change after administration of the poison was compared to the control. The poison was given through a cannula in the femoral vein.

e). The Effects of Arrow Poison on the Heart

Male Sprague-Dawley rats were anesthetized with sodium pentobarbital (30 mg/kg, i.p). The ECG was recorded by coupling the impedance pneumograph coupler to an ECG high-grain coupler(Type

7171, Narco Biosystems, Houston, TX). The setting of the coupler and amplfier was as follows: gain, 3.20 x 100; Filter, 3Hz, the variable scale, 10mV/cm; and the polarity was +. The electrodes on the rat were adjusted in order to get Type II lead recordings. The heart rate was measured and expressed as beats per minute. The ECG waves were analyzed for signs of arrhthmias and/or myocardial depression.

3. Separation of the Active Components

a). Thin Layer Chromatography (TLC)

A 630 mg portion of the poison was weighed and dissolved in deionized water and the resulting solution had a pH of 7.0 with a conductivity of 200 megaohms. Other chemicals used in this investigation were obtained from the following sources: d-tubocurarine, scopolamine, atropine, sodium chloride and sodium hydroxide (Sigma Chemicals, St. Louis, MO.); Dowex 1 x 2 ion exchange resins (Biorad Lans Richmond, CA), all the solvents for TLC (Curtin Matheson, Dallas TX), the reagents for HPLA (Burdick and Jackson Laboratories, Inc. Muskegon, Michigan) and TLC plates coated with silica gel 60F-254 (EM Laboratories, Inc. Elmsford, N.Y. 10523).

Portions of the solution of the arrow poison and similar solutions of atropine, scopolamine and d-tubocurarine were spotted on silica gel plates were then set in different solvent systems to facilitate movement. The solvent systems used were marked 1,2,3,4, and 5 and were of the following composition: ethyl acetate, 2-propanol, 25% ammonia (45:35:5), chloroform, cyclohexane, diethylamine

(3:6:1); butanol, water, acetic acid (12:3:1); ethylacetate, 2-propanol, 25% ammonia (9:7:2) and; methanol ammonia (2.0M) and ammonia nitrate (1.0M) respectively. The solvent front was allowed to migrate approximately 95% of the distance possible before removing each plate. The migration of each other components was identified with the use of UV light. The results of thin layer chromatography was expressed as R_f values.

b). Ion Exchange Chromatography (IEC)

A 170 ml. solution of the arrow poison containing 630 mg. of the original poison was charged on to a 1200 ml. Dowex 1 x 2 column in the formate form. The column was then eluted with the following eluants in sequence: 0.1M MaOH - 0.1M NaCl for 10 column volumes; 0.2M NaCl 0.1M NaOH for 7.2 column volumes; 0.4M NaCl - 0.1M NaOH for 5.3 column volumes and; 1.0M NaOH for 14 column volumes. Ten ml. fractions were collected and the absorbance at 260nM was determined on a spectrophotometer. The results were plotted as absorbance against fraction number.

c). High Performance Liquid Chromatography (HPLC)

The chemical reagents used were purchased from Sigma Chemical Company (St. Louis, MO) or from Burdick and Jackson Laboratories (Muskegon, MI).

The acetonitrile, methanol, chloroform and carbon tetrachloride were used to elute the components of the toxin from the column as received from commercial sources. The sodium chloride solution was made from crystals as required. Appropriate elution solvent mixtures

were prepared as follows: sodium chloride (0.02M)/acetonitrile (8:2); acetonitrile/deionized water (9:1) and; gradient elution of sodium chloride (0.02M)/acetonitrile (45-95%), methanol/deionized water (98:2) and; chloroform and carbon tetrachloride (10-50%) gradient elution.

A 200mg protion of the poison was weighed and dissolved in deionized water and filtered through a millipore filter (pore size, 0.45um). A 20 ul aliquot of the sample was injected into a Perkin-Elmer Series 3 HPL (Perkins-Elmer, Norwalk, Connecticut) equipped with a deuterium power supply, a digital programmer, an optical unit for detection of UV absorption at 260nm, a stainless steel HPLC column (25 x 0.26 cm) packed with Silica-A (HC-ODS-Sil-S, Lot 34) and a model 023 recorder. The flow rate was 1.0 ml/minute (-cm/min.) with a pressure drop of 1800 psig at ambient temperature.

4. Quantification of Arrow Poison

a). Physical Methods

Different sample size of poison (2.0×10^{-8} - 2.0×10^{-5}g) was injected into the HPLC and eluted with deionized water and the resulting response (peak height) was measured. The peak height was expressed as a % of maximum response and plotted against the sample size.

b). Bioassay

Our preliminary data using isolated frog heart according to the method of Straub (1922) and Bulbring's methods have provided us with a sensitivity which is as low as 0.1ug. The two methods are as follows:

i) Preparation of isolated frog heart for the measurement of cardiac contractility

Northern grass frog. Rama papiens, approximately 3½ in. from nose to anus, used in these experiments were purchased from Nasco Biological Science (Fort Atkinson, Wisconsin). The frogs were pithed and the heart was cannulated and removed according to the Strub-Fuehner method (Sollman, 1922). The cannulas contained 1.0ml of Frog Ringer solution of the following composition (mM): NaCl, 111.5; KCl, 1.88; NaH_2PO_4, 0.06; glucose, 11.1; $NaHCO_3$, 4.6; $CaCl_2$ 1.08. Since the reference is old and is not easily available in most libraries, a brief description of the method is provided to help investigators who would like to use the method.

The pericardium of each frog was carefully removed before ligaturing the right aorta. The inferior vena cava, pulmonary artery and vein were also ligatured. The left aorta was cannulated with a glass cannula filled with Frog Ringer solution. The cannula was pushed centrally through the left aorta and the valve into the ventricle. The position of the cannula was considered to be correct when the Frog Ringer solution in the glass cannula was pumped up and down as the heart contracted normally. The heart was then isolated, and set up as shown in Fig. 6a. The blood in the isolated heart was washed out gently by changing the Frog Ringer in the cannula several times. The preparation was allowed to equilibrate for 30 min. at ambient temperature while attached by means of a small clip at the apex of the ventricle to an F-50 microdisplacement isometric myograph transducer (Narco Biosystems, Houston, TX).

The optimum counterweight was determined to be 0.5g. The myocardial contractions were measured and displayed on a DMB-4B physiograph (Narco Biosystems, Houston, TX).

Toxin was applied to the preparation by adding the desired dose into the cannula such that the final concentration was achieved after dilution in the bathing Frog Ringer solution. The action was terminated by changing the bathing medium after the response had stabilized. The procedure was repeated after the contractions were back to pre-drug state, which were considered as control contractions. The response was presented as a percent increase or decrease of the control contractions. The dose-response curve was constructed on semi-logarithmic plots and the ED_{50} for DA and DAA were graphically determined.

ii). Effects of the Toxin on the cardiac Output

After decapitating and pithing each frog, the heart was exposed and the pericardium was removed. All of the blood vessels, except left aorta and inferior vena cava were ligatured. A Marriotte bottle filled with Frog Ringer was connected at the base to a rubber tube leading to a cannula which was inserted into the inferior vena cava. The left aorta was cannulated and the site of outflow of the cardiac output was positioned such that there was a maximum outflow from each contraction cycle of the heart. The experimental set-up is illustrated in Fig. 6b. The preparation was left to equilibrate for 20-30 min at room temperature by which time the cardiac output was consistent.

Cardiac output was measured for a known time period (1 min). The procedure was repeated at least three times and the mean was considered to be control cardiac output. After a control cardiac output was measured, each frog heart was perfused with Frog Ringer containing toxin. Alterations in cardiac output were monitored for each toxin concentration and the changes in cardiac output elicited by the toxin were expressed as a percent of the control.

4. Effect of toxin on the sartorius nerve-muscle preparation

The frogs were decapitated, pithed and the sartorius nerve-muscle was dissected carefully from both legs. The nerve-muscle preparation was mounted to a special holder with platinum electrodes such that the nerve and muscle can be stimulated separately. Each preparation was left to equilibrate for 20-30 minutes at 22°C in aerated (95%O_2 - 5%CO_2) frog Ringer solution. The preparation was then subjected to an initial tension of 0.5g and then left to equilibrate for an additional 30 minutes. The nerve was stimulated with rectangular pulses at 75ms duration and a frequency of 1Hz using a Grass S88 stimulator. The resulting isometric contractions were displayed on a Narco-Biosystems DMP-4A physiograph. After the control contractions were established, the toxin was added to the Ringer solution in the tissue chamber and its effect on the muscle and nerve were recorded.

RESULTS

1. Acute Toxicity Studies

a). Determination of Lethality (LD50)

The arrow poison was toxic to mice with an LD50 of 27mg/kg (i.v). Among the animals dying from a dose of the poison, 90% of them died within two minutes of drug administration; the remaining 10% died within 5 minutes. Dying mice went through a stage of violent excitement, tonic convulsions, and muscle tremors, particularly of the biceps femoris and other hip muscles. In addition, salivation, lacrimation, occasional defecation dyspnea and prostration were observed after injection of a lethal dose of the toxin. The results of acomparison of the posture of mice dying from a lethal dose of the poison, epinephrine and d-tubocurare suggested that muscle paralysis was part of the cause of death (Fig. 2).

b). The Effect of cholinergic Antagonists on LD50

Since some of the symptoms of the acute toxicity of the poison in mice appeared to be mediated through the cholinergic system, we also investigated the effects of cholinergic agents on the LD50 of the arrow poison in mice. Pretreatment of the mice with cholinergic agents appeared to decrease the lethality of the poison (Table I).

c). The Effects of Sex on the LD50

Our results indicate that sex does not have any significant effect on the LD50 of the poison (Table II).

d). The Cardio-respiratory Effects of the Poison

The poison initially reduced the respiratory rate and the

heart rate of rats *in vivo*. The onset of action was very rapid and the respiratory rate came to a complete stop within 10.0 ± 1.2 (N =6) seconds of administration of the poison. Similarly, the heart rate was decreased and eventually came to a complete stop within five minutes. The recordings of respiration and heart rate are presented in Fig. 3.

2. Separation of the Active Components

a). Thin Layer Chromatography (TLC)

The results of TLC on silica gel are presented in Table III. Of the different solvent systems used, the components of the arrow poison appear to either have not moved or only moved in solvent system 4 which was composed of ethylacetale, 2-propanol, and 25% ammonia (9:7:2). It is significant to note from the table the lack of similarity between the mobility of the poison and d-tubocurare.

b). Ion Exchange Chromatography (IEC)

The application of IEC in the separation of the posion with gradient elution of NaCl (0.1 - 1.0 M) and NaOH (0.1M) resulted in the successful separation of the active components into three distinct peaks (A,B and C in Fig. 4). Each of these peaks have been shown to be homogenous except for peak C which came out as a doublet when subjected to further separation using HPLC (data not shown).

c). High Performance Liquid Chromatography (HPLC)

The results of the separation of active principle(s) using HPLC with the elution profile using a gradient with 0.05 NaCl and acetonitrile ranging in concentration from 45% to 95% (v/v) are

presented in Fig. 5. According to the figure, the poison can be resolved into four distinct peaks. Other tested solvent did not resolve the toxin into more than three peaks and the results are therefore not presented.

3. Quantitation of the Arrow Poison

a). Physical Method

Since the separation of the poison with HPLC using deionized water as a solvent led to the elution of only one peak (Fig. 5a), we decided to use this solvent to investigate the relation between the sample size injected into the column and the peak height. The relationship is linear (Fig. 5b) and is useful in quantitating the arrow poison.

b). Bioassay

Our preliminary data using isolated frog heart according to the method of Straub (a922) has provided us with a sensitivity which is as low as 0.1ug. The arrow poison dose-dependently decreased the cardiac contractility and the cardiac output of the frog heart. The dose range for the effect on contractility was 1 - 10mg/ml. The relationship between the concentration of the poison and the decrease in cardiac contractility was linear suggesting the potential for the use of the technique for bioassay of the toxin. The low concentration of the toxin (0.2 - 20mg/ml) increased the cardiac output, however, at high concentration (>200ug/ml) the toxin decreased the cardiac output in a dose-dependent fashion.

4. Effect of the toxin on the isolated sartorius muscle of the frog

The toxin suppressed the neuromuscular transmission of the satorius nerve-muscle preparation (Fig. 7). According to the recording the direct effect on the muscle was not as great as the one on the nerve.

DISCUSSION

Exploration of folk medicine as a source of desirable therapeutic agent (s) is a difficult task requiring: systemic toxicological and pharmacological studies to provide basic reference information against which the isolated fractions can be compared; separation of the active components without loss of chemical activity; quantitative methods; determination of structure of the active components and; synthesis of the isolated active components for further toxicological/pharmacological and metabolic studies.

The results of our acute toxicity studies of the arrow poison indicated that the toxin kills the prey through muscle paralysis and cardio-respiratory collapse. The magnitude of the significance of the peripheral effects of the arrow poison e.g. salivation, urination, lacrimation etc. on the lethality of the poison cannot be deciphered on the basis of our data since classical anticholinergic agents did not completly protect the animals from the toxin. However, the pretreatment of the mice with anticholinergic agents decreased the lethality (Table 1). The cholinergic systems appears to be involved in the mechanism of toxicity of the arrow poison. The sex of the animal did not appear to have any significant effect on the lethality of the poison.

The most significant part of the results of TLC is that the mobility of the poison (F value) was completely different from that of d-tubocurare (Table III). Generally, d-tubocurare is considered

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c). The Effects of Sex on the LD50

Our results indicate that sex does not have any significant effect on the LD50 of the poison (Table II).

d). The Cardio-respiratory Effects of the Poison

The poison initially reduced the respiratory rate and the

the prototype active component of arrow poisons of plant origin (Koelle, 1970). However, the results of our observation of the mice dying from lethal doses of d-tubocurare, epinephrine and the arrow poison suggest that d-tubocurare and arrow poison kill the mice at least in part through muscle paralysis (Fig. 2). The resolution of the arrow poison into different components was very poor. Therefore, we decided to try other chromatographic techniques to separate the toxin.

IEC with gradient elution was a better technique than TLC as shown in Fig. 4. The peaks are distinct and easily identifiable. However, the method is tedious and time-consuming. Therefore, we tried HPLC to separate the active components of the toxin since HPLC has been established to be rapid and efficient (Garnier et al. 1979).

Different solvents and their mixtures were tried in the HPLC to separate the active components of the arrow poison. The best separation was achieved using gradient elution with 0.05M sodium chloride acetonitrile (45-95%) which resulted into the resolution of the poison into four distinct peaks (Fig. 5). In order of decreasing efficiency, the quality of separation achieved with the different solvent tried was as follows:
gradient elution with 0.05M sodium chloride/acetonitrile (45 -95%) methanol and deionized water (98:2); acetonitrile and deionized water (98:2); gradient elution of chloroform/carbontetrachloride (10-50%); sodium chloride (0.02M) and acetonitrile (8:2); deionized water. The results with deionized water as the solvent was unique in that the

poison was eluted as one single peak.(Fig. 6a). This unique observation provided the basis of choosing deionized water as the ideal solvent for using HPLC to quantitate the arrow poison.

The relationship between the sample size and the peak height was linear for the toxin size ranging from 20ng to 20mg (Fig. 6b). This observation provide support for the use of HPLC as a powerful tool for the separation and quantifying the arrow poison and its active components. The sensitivity of the bioassay method using isolated frog heart in vitro and also frog heart in situ provide techniques which are sensitive enough for the bioassay of the toxin. We believe that these techniques and the HPLC method provide sufficient methods to standardize the crude toxin and to investigate further the isolated active components.

Table I. Acute toxicity of arrow poison in mice. The details about the procedure are presented under materials and methods.

TREATMENT	LD50 (mg/kg)
TOXIN	26.89 ± 0.73
PHYSOSTIMINE + TOXIN	39.0 ± 1.0
ATROPINE + TOXIN	32.5 ± 2.39
d-TUBOCURARE + TOXIN	42.0 ± 12
HEXAMETHONIUM + TOXIN	30.16 ± 3.57

Table II. Influence of sex on acute toxicity of arrow poison in mice. The details of the experiment are presented under material and methods.

SEX OF MICE	LD50 (mg/kg)
MALE	26.58 ± 0.93
FEMALE	27.5 ± 1.32

Table III. Results of Thin Layer Chromatography on Silica Gel. The spots were one application per spot.

Solvent System	Agents spotted on silica gel plates and their respective R_f values.			
	Atropine	Arrow Poison	d-Tubocurarine	Scopolamine
1	0.368	0	0	0
2	0.330	0	0.71	0.60
3	0.720	0	0	0
4	0	0.90	0.57	0.60
5	0.06	0	0.06	0.06

* The solvent systems marked 1,2,3,4, and 5 are of the following composition. Ethyl acetate, 2-proponol, 25% ammonia (45:35:5); chloroform, cyclohexane diethylamine (30:60:10); butanol, water, acetic acid (60:15:5); ethylacetate 2-proponol, 25% ammonia (45:35:10); methanol ammonia (2M) and ammonium nitrate (1M), respectively.

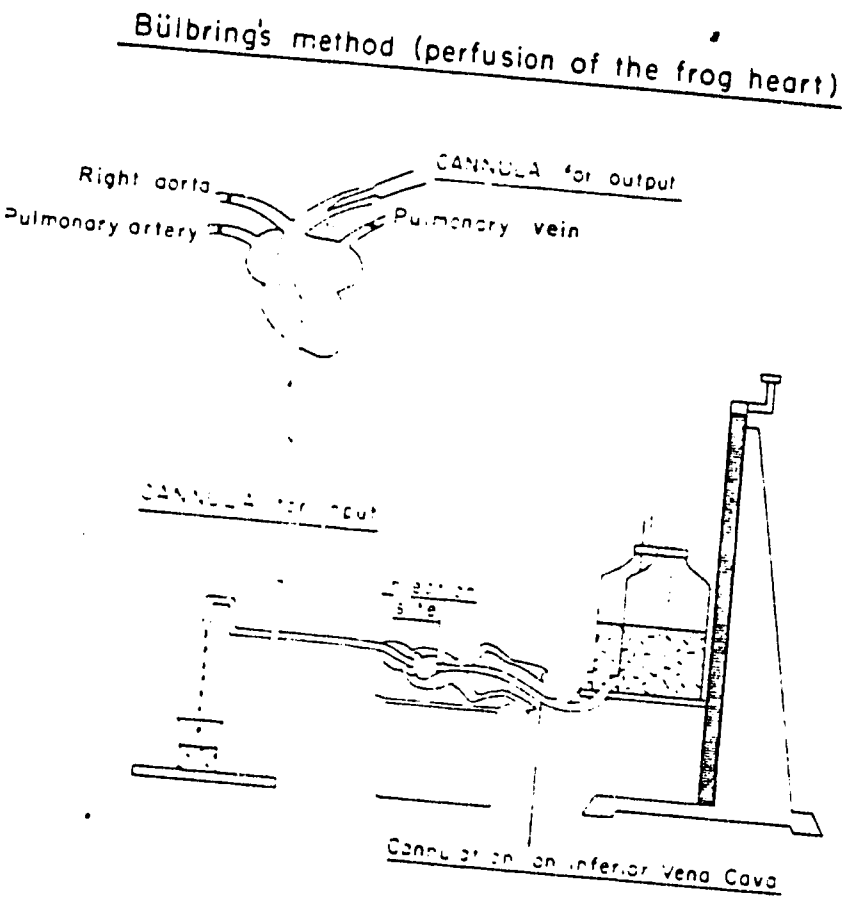

FIGURE 1b

Schematic diagram for the procedure of cannulation and setting it up for recording cardiac output. In this experiment the toxin was added upto the desired concentration in the Marriotte bottle instead of applying the drug at the injection site.

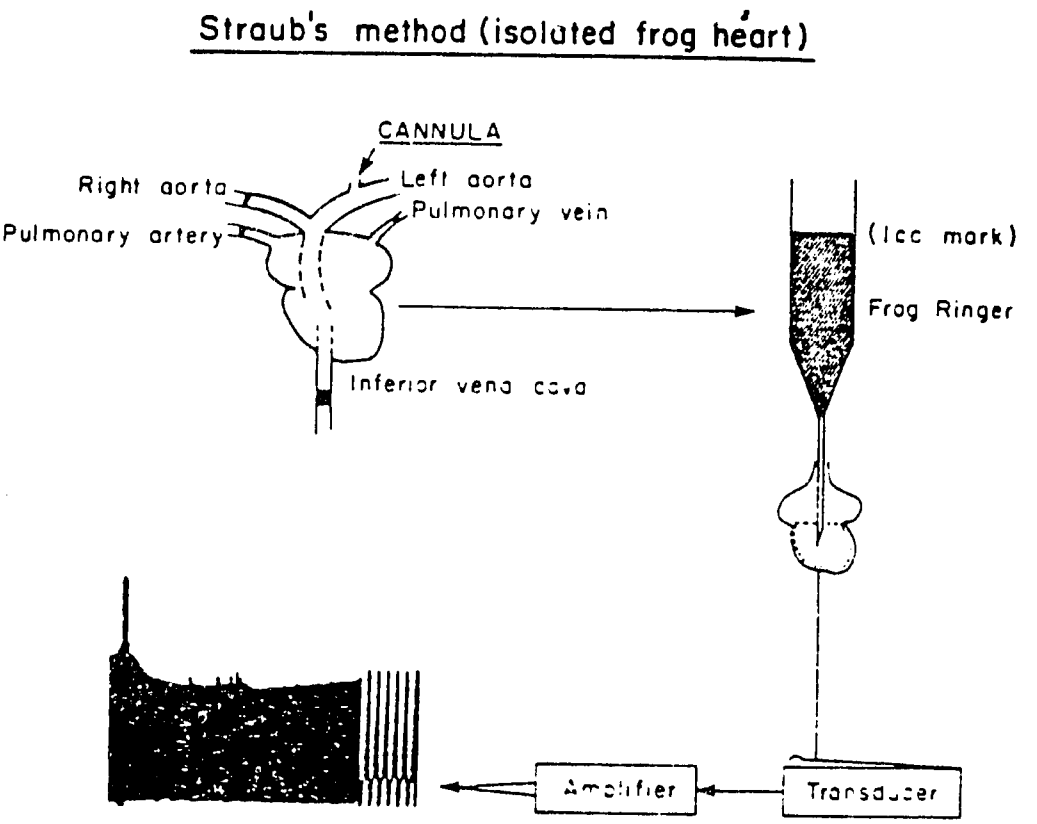

FIGURE 1a

Schematic diagram for the procedure of cannulation, isolation of frog heart and setting it up for recording the effects of toxin on cardiac contractility.

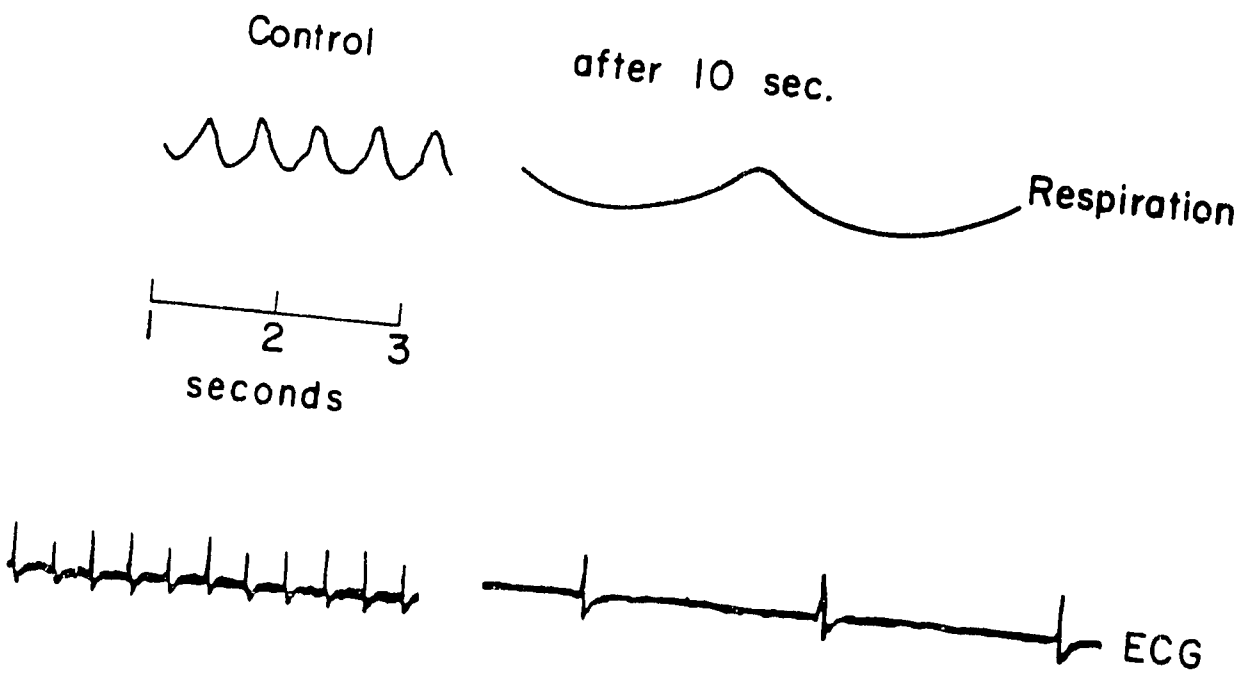

FIGURE 3

The cardio-respiratory effects of an African arrow poison in male Sprague Dawley rats. The respiratory rate and ECG were recorded by coupling the Hi-Gain coupler with impedence pneumograph coupler which was connected to the rat through a Lead II connection. The dose of the drug used was a lethal dose (For details see text). The effects of the poison was prominent within 10 seconds of drug administration.

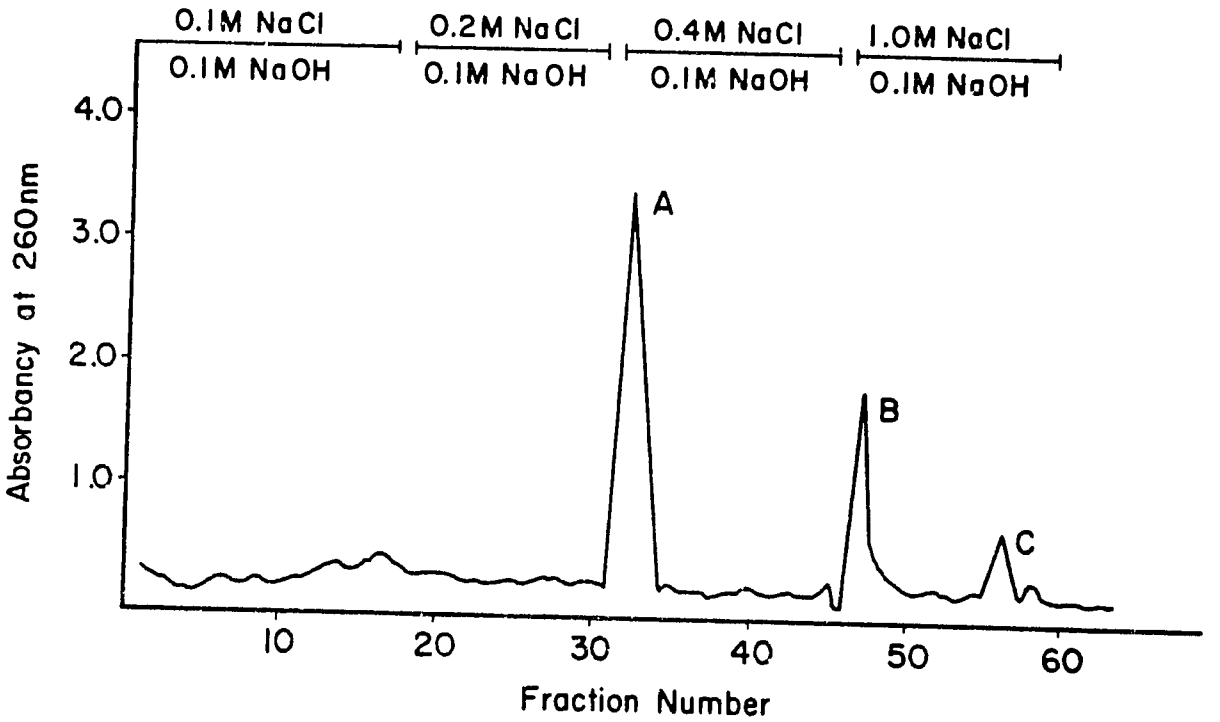

FIGURE 4

Dowex 1 x 2 ion exchange chromatography of 630 mg of crude arrow poison on a 2.5" x 33" column with step gradient elution.

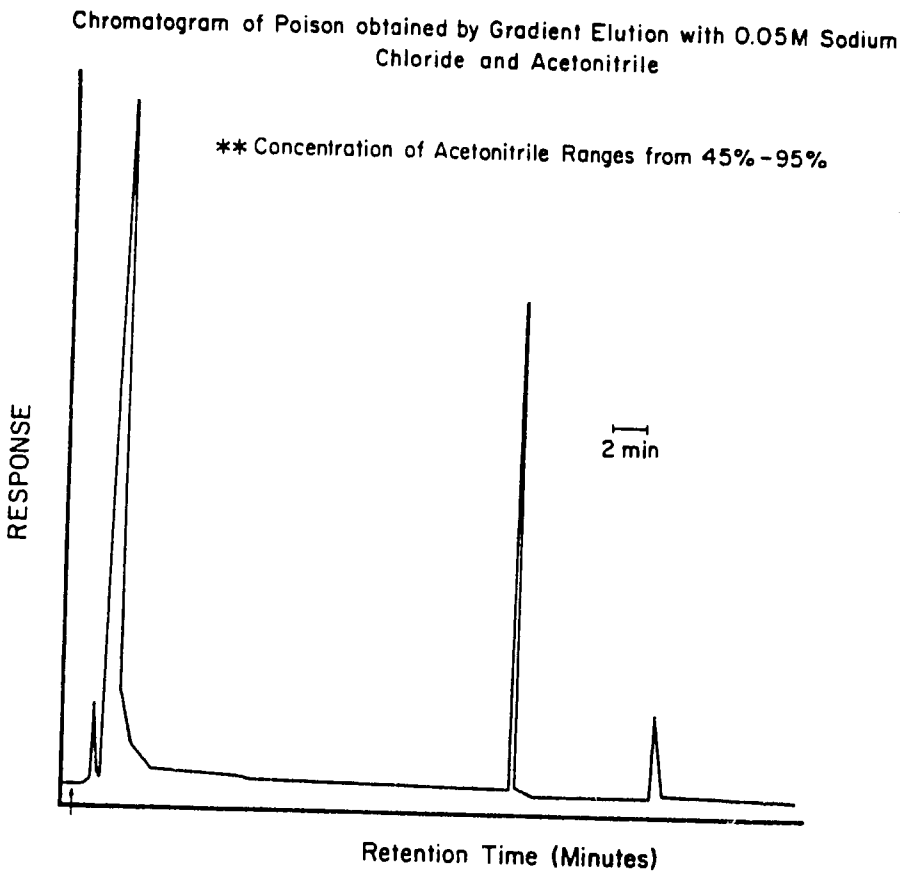

FIGURE 5

The elution profile using a gradient with 0.05 sodium chloride and acetonitrile ranging in concentration from 45% to 95% (v/v).

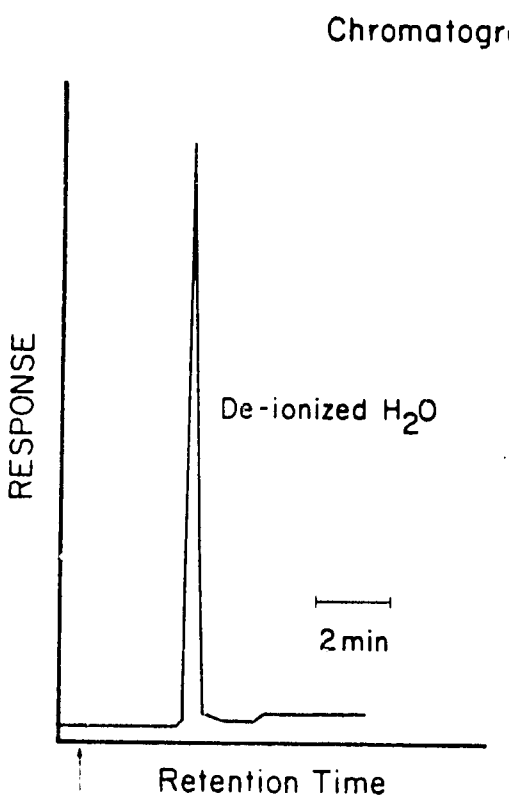

FIGURE 6a

Chromatograms of the eluted peaks of the arrow poison as a function of retention time. The solvent used for elution was deionized water which had been established to elute the toxin as one singlet making it ideal for quantitation based on peak height.

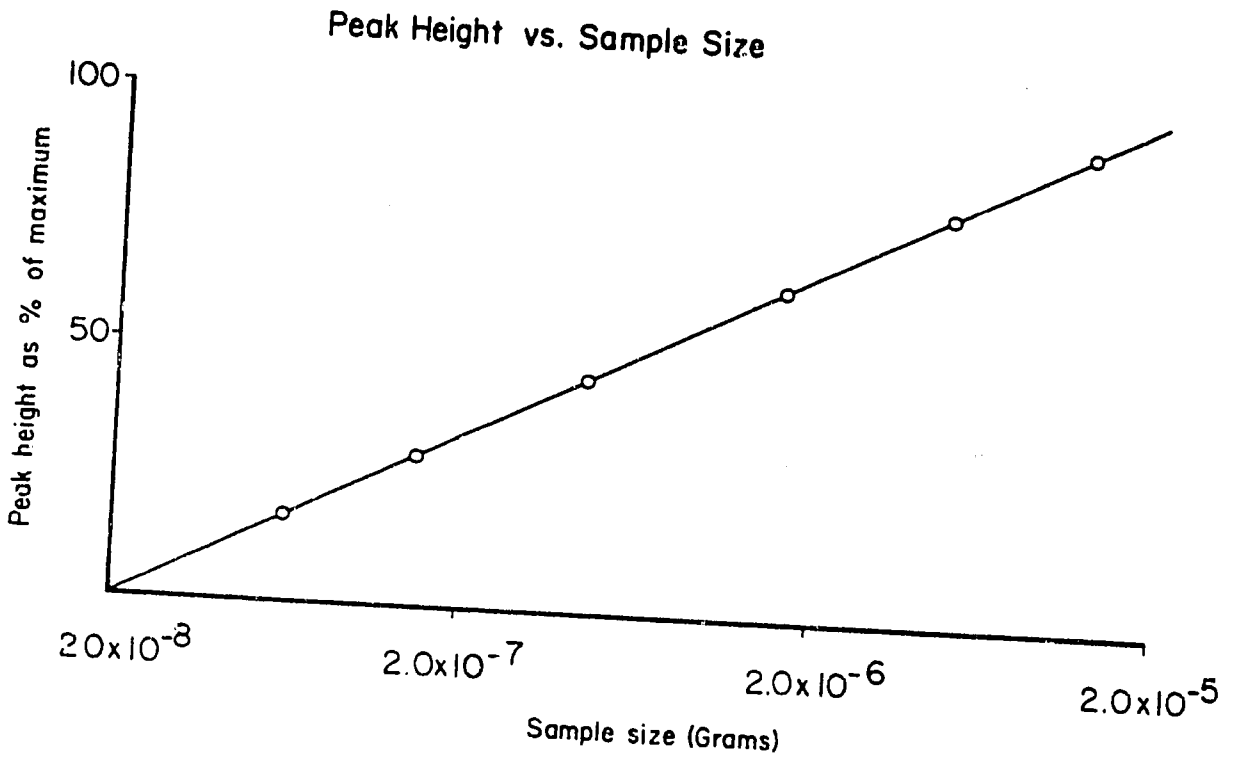

FIGURE 6b

Calibration curve for solution of the arrow poison.

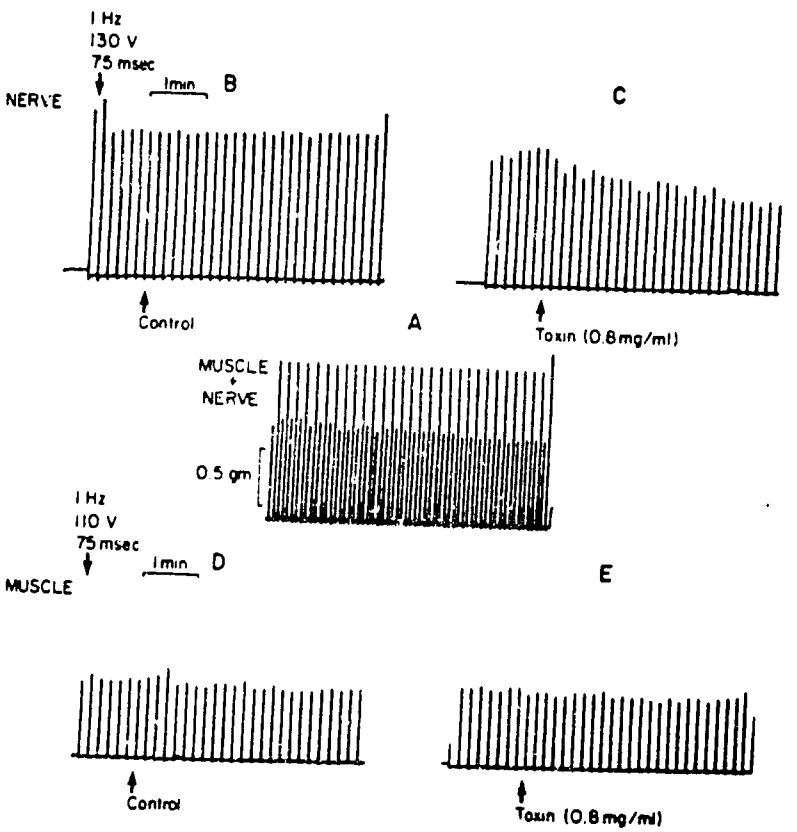

FIGURE 7

The effect of the toxin on the isolated satorius muscle and nerve preparation. The parameters for the electrical stimulus and the dose of the toxin is shown in the figure.

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