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A method of analysis for saxitoxin (STX) in the rat (blood serum and urine) and human urine was developed using CBA and EMPORE columns followed by oxidation with hydrogen peroxide and analysis by HPLC using a C18 reversed phase column and detection by fluorescence of the OPA derivative. Detection was also made by continuous flow fast atom bombardment (CF/FAB/MS). STX was found in the serum and urine of rats treated with STX (1 to 3 µg total intraperitoneal injection) as soon as 10 min after dosing and up to 8 hrs. Rats dosed with 75 µg by stomach intubation had detectable amounts of STX in their urine for up to 31 hrs. Confirmation was done by CF/FAB/MS using selected ion monitoring (m/z+ 300 & 282). The limit of detection by HPLC was 4 ppb in human urine and 220 ppb in rat urine. The limit of detection by CF/FAB/MS was 25 ppb in human urine. A method of analysis using EMPORE column technology yielded a percent recovery of 102 at 25 ppb and 99 at 50 ppb. CBA columns used for comparison gave a percent recovery that varied between 64 and 90%.

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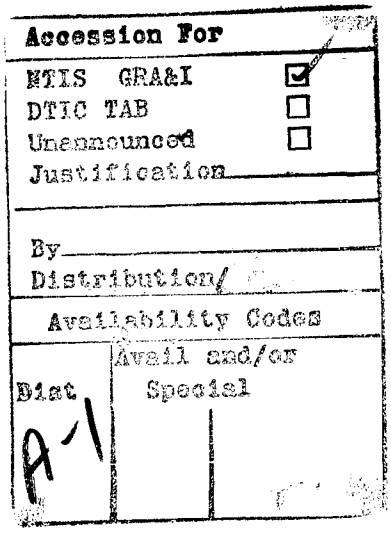

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KINETIC STUDY OF THE EXCRETION OF SAXITOXIN IN RATS TREATED WITH SUBLETHAL AMOUNTS OF SAXITOXIN

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SUMMARY

A method for analysis of saxitoxin (STX) in urine of rats treated with STX was developed using CBA columns for cleanup of the extract and hydrogen peroxide oxidation prior to analysis by HPLC using fluorescence of the OPA derivative of STX. Detection of STX is linear between 0.1 and 5 ng when measured by either peak height or area. It was used for analysis of urine from rats treated with 1.5 ug total STX. The limit of detection of pure standard is 100 pg.

Saxitoxin was found in the blood serum of rats treated by intraperitoneal injection (i.p.) with a total dose of 1.5 ng STX. It was detected as soon as 10 min after treatment and up to 24 hrs after dosing. The largest amount of STX (10-16 ng/ml) can be found 1 hr after treatment followed by small (2-4 ng/ml) amounts of STX up to 24 hrs. Rats dosed i.p. with 1 ug STX had detectable amounts of STX in their urine up to 8 hrs after treatment (termination of the experiment). The excretion pattern was bimodal with the greatest amount (181 ng/ml) recovered 1 hr after treatment followed

by smaller amounts (75 to 95 ng/ml) 4 to 6 hrs after dosing. Rats treated with 75 ug STX by stomach intubation had detectable amounts of STX in their urine up to termination of the experiment (31 hrs). In general, the excretion pattern of STX in urine was bimodal.

Adult female rats tolerate a 1 ug dose of STX given by intraperitoneal injection but succumb to 3 to 4 ug (i.p.) within 3 min.

Confirmation of STX in both rat and human urine was done by continuous flow fast atom bombardment mass spectrometry (CF/FAB/MS) using HPLC and microcapillary columns. The confirmatory ions are found at m/z+ 300 (299+H)+ and 282.

INTRODUCTION

Saxitoxin was first isolated and described from toxic Alaska butter clams known by the Latin binomial **Saxidomus giganteus** (Schantz et al., 1957). The paralytic shellfish poisons (PSP) include STX which is one of the most serious marine toxins known (Hashimoto and Noguchi, 1989). Ingestion can cause paralysis often terminating in death. **Protogonyaulax catenella** produces STX and grows to a high density under favorable environmental conditions. The organism is ingested by bivalves which, when consumed, may in turn poison humans (Sommer, 1937).

Various analytical methods have been devised including high performance liquid chromatography (HPLC) separation (Sullivan and Wekell, 1987), capillary zone electrophoresis (CZE) (Thibault et

al., 1991a and b), continuous flow fast atom bombardment (CF/FAB), (Caprioli, 1989), and ion spray mass spectrometry (IS/MS) (Quilliam et al.,1989). Perhaps the most useful and inexpensive is the HPLC method described by Lawrence et al.(1991) where STX was oxidized with hydrogen peroxide prior to separation on the HPLC column and detection by fluorescence. The limit of detection is 100 pg and the limit of sensitivity of the method is about 100 ppb. The oxidation procedure takes 10 min and analysis by HPLC takes another 10 min. This method adapts itself to analysis of biological fluids and is the primary determinant of success in this study.

There are numerous reports on the effects of STX and ion transport channels but little to none on metabolism in living animals or detection in the urine of treated animals. As an example, Xia and Haddad (1993) studied the neuroanatomical distribution and binding properties of STX in the rat brain but offered no metabolic data. They concluded that adult rat brain has multiple sodium channels but similar binding affinities for all of them i.e. little selectivity. Fan and Makielski (1993) studied STX blockage of sodium channels in cardiac myocytes in the rat but no metabolism studies were reported. The study of binding sites and STX "dwell time" in the animal is important for studies of metabolism because STX can be found in the urine almost instantly (as fast as one can collect the urine) after insult. The questions remain as to what absolute amount is needed to bind and affect the rat and whether there is an activated metabolic species that actually binds to the receptor.

Our objectives in this study were (i) to study the detection of STX in the rat, using urine as the primary determinant, in order to verify exposure to STX; (ii) to identify any metabolic products that may be present other than the parent metabolite for purposes of monitoring exposure; and (iii) to develop a method of extraction and detection of STX in urine.

MATERIALS AND METHODS

Collection of rat urine after dosing with STX: The rats used in the experiment are Sprague Dawley, female, 9 to 10 months old and weighing 300-350 grams. They were provided with a standard rodent ration and water. Three rats were dosed with saxitoxin dihydrochloride (STX.2HCl) using water as the carrier solvent. One microgram of STX in 1 ml water was injected into the intraperitoneal cavity at zero time and then once more after 24 hrs. The rats survived the treatment and urine was collected after 1, 2, 3, 4, 6 and 8 hrs on the first and second day treatments. The urine was collected in a clean Petri plate by gently holding the rat in the hand over the plate and softly squeezing the mid section of the rat. On the third day, the rats were dosed once more, but this time with 4 ug. All rats died within 3 min after administration of the third dose. Urine was collected within 10 minutes directly from the bladder and stored in a freezer. Urine from nontreated rats (controls) was collected in the same manner and kept frozen.

Preparation of serum from rats dosed with STX: Sprague Dawley rats were treated with 1.5 ug STX by intraperitoneal injection as described above and sacrificed by asphyxiation with CO2. The whole

blood was collected by cardiac puncture immediately after expiration. The blood was placed in 15 ml centrifuge tubes and gently stirred with a wooden spatula and then allowed to stand at room temperature for 2 hrs. The blood was then placed in a refrigerator and the serum allowed to separate. The next day the blood was centrifuged at 7000-8000 rpm at 5 C for 10 min to obtain the supernatant serum.

Oxidation procedure for the analysis of STX by HPLC: Analysis of STX using the old method of postcolumn oxidation and detection by HPLC as described by Sullivan and Wekell (1987) is cumbersome. An alternative method is precolumn oxidation as described by Lawrence et al. (1991) in which the oxidation is performed under controlled conditions in sample preparation prior to injection into the HPLC apparatus. Two oxidation procedures were described, periodate and hydrogen peroxide. The method of choice for STX oxidation is hydrogen peroxide.

The method described by Lawrence et al. (1991) involves oxidation of STX at room temperature with hydrogen peroxide. The rat urine sample was first prepared by passing through a CBA column followed by concentration and oxidation.

CBA column procedure: The CBA column was purchased from Analychem International, Los Angeles, CA. It is a carboxymethyl (hydrogen form) sorbent and a weak cation exchanger. Its pKa is 4.8 and thus it is useful for resolution of STX. The CBA bed volume is 100 mg; it is conditioned with 4 ml of methanol followed by 4 ml of

0.05 M KH2PO4/Na2PO4 (pH 8.0). To 0.1 ml sample of urine, 2 ml of 0.05 M KH2PO4/Na2PO4 (pH 8.0) was added, thoroughly mixed and the mixture loaded onto the CBA column. The column was washed with 5 ml water followed by 2 ml of methanol. The STX fraction was eluted off the column with 2 ml of 20% aqueous acetic acid. The eluate was taken to dryness under nitrogen without heating.

The blood serum (1.5 ml) was mixed with 4 ml of a mixture of 0.05M NaH2PO4/Na2HPO4 (pH 8). The mixture was loaded onto a CBA column followed by washing with 5 ml water and 2 ml methanol. The STX was eluted off the column with 2 ml aqueous 20% acetic acid.

Oxidation with hydrogen peroxide: The dried sample was dissolved in 100 ul of water to which was added 25 ul of aqueous hydrogen peroxide (10% w/v) and 250 ul of 1 N NaOH. The sample was mixed thoroughly and allowed to react for 5 min at room temperature (20 C) A 25 ul volume of concentrated acetic acid was added and mixed thoroughly. Twenty microliters of the solution was injected into the HPLC (Shimadzu) system.

Detection by HPLC: The instrument used for HPLC analyses was a Shimadzu SLC-6A (Shimadzu Co. Kyoto, Japan) equipped with a Shimadzu RF-530 fluorescence monitor. The excitation wavelength used was 330 nm combined with a monitoring wavelength of 410 nm. The column used was a C18 reversed phase (Waters NOVA-PAK) 75 mm x 3.9 mm i.d.. The HPLC mobile phase consisted of A = 0.1 M ammonium formate in water (adjusted to pH 6 with acetic acid) and mobile phase B= 20% acetonitrile in mobile phase A (pH 6). Solvents A & B

were run from 0.1 to 14 min with A at 1-0.7 ml/min and B at 0-0.3 ml/min. The retention time window for STX on the column was 9.3 to 9.76. The chromatographic procedure was run at room temperature i.e. 23 to 25 C.

Percent recovery: Experiments were done by spiking 0.1 ml rat urine with 0.1 ml aqueous solution containing 100 ng STX. The latter was mixed with 2 ml buffer at pH 8.0 and loaded onto the CBA column. The recovery was 95.5%. In another experiment, 2 ng of STX was spiked into 0.1 ml rat urine and then mixed with 2 ml of buffer (pH 8) and loaded onto the column. The recoveries in 2 experiments were: 100% and 110%; average 105%.

Standard curve: One-tenth milliliter of standard STX solutions (20 ng/ml, 200 ng/ml and 1 ug/ml) was mixed with 25 ul of aqueous H2O2 (10%), 250 ul 1N NaOH (react for 5 min at room temperature) and 25 ul acetic acid (concentrated) to stop the reaction. Twenty microliters of this mixture was injected into the HPLC. The latter injection volume gave 0.1, 1.0 and 5.0 ng oxidized STX as shown in Figure 1.

Microcapillary chromatography of STX by continuous flow fast atom bombardment (CF/FAB/MS): CF/FAB/MS was done on a VG 7070EQ, hybrid, tandem mass spectrometer equipped with a CF/FAB probe tip and xenon gun from Fisons (VG Analytical Ltd), Manchester, England. The FAB gun was run at 8 Kv. A Shimadzu (Shimadzu, Inc., Kyoto, Japan) LC-600 dual piston pump and an Applied Biosystems 140B Solvent Delivery System (both capable of delivering a solvent at 1

ul/min) were used in conjunction with a 50 micron internal diameter (i.d.) fused silica column threaded to the flat probe tip in the source. Injections were made with a Rheodyne (Cotati, CA) injector with 200 or 500 nl rotor or a Valco (Valco Instruments, Inc., Houston, TX) 60 or 200 nl injector. The microcapillary HPLC column (packed in our laboratory) was 20 cm in length by 320 microns i.d. packed with 3 micron particle size C18 reversed phase packing. The mobile phase solvent systems used were: A= 2% glycerol, 98% 0.01M pentafluoropropionic acid (PFPA; B= 2% glycerol, 98% 0.01M PFPA, 20% acetonitrile. The total flow rate was 180 ul/min and the column flow rate was 4 ul/min; the split ratio was 45:1. The gradient program consisted of 100% "A" solvent for 1 min changing to 100% "B" solvent system in 14 min. This was followed by the "A" solvent system for 5 min.

RESULTS AND DISCUSSION

Oxidation of STX: The fluorescent single oxidation product of STX as well as oxidation products of other paralytic shellfish poisons (PSPs) were described by Janecek et al. (1993). Quilliam et al. (1993) described the mass spectrometry of the oxidation product (protonated molecular ion in FAB is 296) and its fragmentation product (m/z 235). A standard curve based on fluorescence of the oxidized product is shown in Figure 1. The oxidation product is stable to at least one-half hour; we inject the mixture into the HPLC after 5 min. There is a linear relationship between the three concentrations tested, i.e., 0.1, 1.0 and 5 ng. The limit of detection is ~100 pg. This is truly amazing because it approaches

the 15 pg limit of IS/MS reported by Thibault et al. (1991a and 1991b) and 50 pg by Mirocha et al. (1992). An overlay chromatogram showing the detector response (fluorescence) to various amounts (0.1, 0.5, 1.0 and 5.0 ng) is shown in Figure 2. The same linearity is shown in the detector response as in the peak height plot. Note the unknown component present at retention time 4.5 in the standard. The unknown may be a STX derivative isolated with the parent compound or a by-product of the peroxide oxidation. It is always present in a constant ratio (10:1) to STX both in the standard and the rat urine isolation products and is shown Figure 2.

STX in blood serum of adult rats dosed with STX: The concentration of STX found in the blood serum of rats dosed with STX by intraperitoneal injection is shown in Table 1. Two rats were used for each collection period, i.e., each rat was sacrificed and the blood collected and saved as described. A total of 18 rats were in the experiment. The STX concentration in ng/ml is shown for each rat as well as the average. The greatest concentration in ng/ml was found in rats sacrificed 12 min after injection. Analysis of the serum collected for the rest of the experiment showed a concentration that ranged between an average of 1.8 and 4.0 ng/ml. The concentration of STX after the first hour of treatment remained more or less uniform until termination of the experiment. The experiment showed that it is possible to find STX in the serum of circulating blood up to 24 hrs after exposure,i.e.,intraperitoneal injection.

The data shown in Table 1 are also presented as a bar graph in

Figure 3. The STX remained at low levels for up to 24 hrs after treatment, indicating that small amounts of STX remain in the circulating blood for an extended period of time. The values of replicates for each treatment agreed rather well considering that each rat actually constitutes a separate experiment. Detection depends on the sensitivity of the analytical method. Although STX can be found in blood serum for up to 24 hrs (end of test period), it is not as sensitive a determinant of intoxication as analysis of the urine.

Two rats were dosed (intraperitoneal injection) with 3 ug total of STX and expired within 10 min from the effects of the toxin. The STX concentration in the serum after 10-12 min were 92.7 and 84.3 ng/ml, respectively. STX can be found in both the urine and blood serum immediately after injection.

Detection of STX in urine of adult rats dosed (intraperitoneal injection) with STX: The collection of urine from live rats is a difficult art and often unpredictable as to the amount that can be collected at any one time. The alternative is to insert an indwelling catheter, which in itself is traumatic to the animal and may compromise metabolism and function of the excretory system. In these experiments, the amount of urine collected from each rat varied sometimes due to the excretion volume and other times due to accidents in collection. The urine was collected in a clean Petri plate by gently holding the rat in the hand over the plate and softly squeezing the midsection of the rat. The collection technique was unique and was done exclusively by one member of our

group who had exceptional skills and patience at performing the operation. The collection time is noted in each table and figure. The urine was frozen immediately and stored for 1 to 2 weeks before analysis. The pH of the urine varied between 6.0 and 6.5. STX is stable at this pH and will tolerate freezing.

Rats 5, 6 and 7 (weight ~300-350 g) received a total dose of 1 ug STX per rat (i.p.) on days 1 and 2 and tolerated this dose very well i.e., there were no external signs of trauma. However, 3 or 4 ug STX resulted in death within 3 min. The LD50 (i.p.) in an adult rat is 8 ug/kg whereas by stomach intubation it is 200 ug/kg for a young rat and 500 ug/kg for an adult rat (Watts et al. 1966). In estimating the quantitative data with STX, one is always left with the question of purity because the sample size available is small and difficult to quantitate.

Urine of rats 5, 6 and 7 was collected on day 1 at 1,2,3,4,6 and 8 hrs after dosing. The rats were dosed once more at 24 hrs and the same collection schedule was followed as on day 1. The quantitative results (expressed in nanograms per milliliter) are shown in Table 2. In general, the majority of STX was found in the 1 hr posttreatment urine (average of 182 ng/ml) followed by a decrease at 2 and 3 hrs (average of 22 ng/ml) and then an increase at 4 and 6 hrs (average of 64 ng/ml),i.e., bimodal excretion pattern. Rats 6 and 7 were dosed with 4 ug STX on day 3 and they succumbed within 3 min. It is important to note that STX was found in their urine (average of 631 ng/ml) almost instantly after injection. A bar graph presentation of these data is shown in Figure 4.

The HPLC tracing of the oxidized product of control rat urine is shown in Figure 5-A. The peak at retention time 10.0 is an unknown component present in control rat urine. Figure 5-B shows the HPLC tracing of the oxidized STX product (retention time 9.7) in rat urine as well as the unknown component at 10.0. The two components are shown in an overlay of the two chromatograms (Figure 5-C) to show the difference in retention time of STX and the unknown. The two are easily resolved and do not present a problem in detection and quantitation.

The total STX recovered, expressed in nanograms, is shown in Table 3. The data suggest that with as little as 1 ug of injected STX, the toxin can readily be detected. On day 1, 236 ng or ~24% was recovered in the urine. On day 2, 91 ng or 9% was recovered. Rats 6 and 7 were injected with 4 ug on day 3; 0.07% of the dose was recovered at time of death, i.e., 3 minutes after exposure.

A graphic representation of the data shown in Table 2 is displayed in Figure 4, where the average concentration of STX is normalized in units of nanograms/milliliter (ppb) for all 3 rats. The day 1 presentation of data shows the bimodal curve; however, data from the second injection on day 2 do not show an immediate excretory response as on day 1 but do show an increment rise in concentration from 2 to 4 hrs. The same pattern of excretion is shown in rat 7 (Figure 6) indicating that perhaps once the animal is insulted with STX, the detoxification mechanism is compromised and does not react at the same speed with the second dose. The best

agreement in recovery of STX is found in rats 5 (Figure 7) and 7 (Figure 6) where 284 and 226 ppb respectively were found. The amount of STX found in rat 6 on day 1 (figure 8) was 34 ppb, which is less than the others; however, although smaller, the pattern of excretion or ratio is similar. The above data can also be expressed in absolute amounts detected,i.e., before normalization to 1 ml, in each rat; the latter are presented in Table 3.

The concentration of STX (ng/ml) found in the urine of rats 6 and 7 dosed with 4 ug STX on day 3 of the experiment is shown in Figure 9. The animals died 3 to 5 min after injection; STX could be found in the urine almost immediately,i.e., the time that it took to make the collection. The speed at which STX is excreted is significant and suggests that a rapid clearance mechanism exists in the rat.

STX in urine of rats dosed with 75 ug STX by stomach intubation: Rats tolerate large doses (75 to 100 ug total) of STX when administered by stomach intubation whereas injection of 3 ug STX intraperitoneally will result in death within 3 min. Each of 3 rats were dosed with 75 ug STX and their urine was collected at the time intervals listed. Each rat is counted as a separate experiment and the results are listed separately in Figures 10-12. Moreover, each rat is an individual in the sense that individual variation and resistance exist and are unique to each animal. Hence, although the results are similar, there are differences in quantity of STX recovered from the urine. Rat 1 shown in Figure 10-A had 53 ng/ml of STX at 0.5 hrs after treatment. There were bimodal peaks of

recovery of STX: at 2 hrs, 278 ng/ml was found followed by a second peak at 27 hrs (416 ng/ml). The concentrations at the other collection times ranged between 27 ng/ml and 201 ng/ml. We have no explanation for the relatively large amount of STX found 27 hrs after treatment. However, it is significant that STX could be found as long as 31 hrs after treatment. Perhaps STX would survive longer but no collection was taken beyond this point. The absolute amounts of STX in nanograms are shown in Figure 10-B.

The concentration of STX in rat 2 after treatment with 75 ug STX by stomach intubation is shown in Figure 11-A. The pattern of STX found in the urine differs somewhat from that found in rat 1 although both show detectable amounts up to 31 hrs i.e. the end of the experiment. The amount of STX peaked in rat 1 at both 2 and 27 hrs whereas in rat 2 it peaked at 4 and 7 hrs. We expected variation in the quantitative results. However, the important finding is that all the data agree that STX can be found in the urine up to 31 hrs after insult. The cumulative percent recovery of STX in rats 1, 2 and 3 were 0.96, 10 and 0.97 respectively. The absolute amount of STX in nanograms found in rat 2 is shown in Figure 11-B

The concentration of STX in the urine of rat 3 is shown in Figure 12A-B. The highest concentration of STX was found 1 hr after treatment and again at 6 hrs. Perhaps the most significant finding was that at 1 hr. after treatment, 5.7 ug/ml or 199 ng absolute amount of STX was found, the highest of the rat test series. Consult Table 4 for urine volumes and concentrations. A summary of the data on rats 1, 2 and 3 is found in Figure 13.

Table 5 shows the urine volume, concentration of STX (ng/ml) and the absolute amount of STX found in the rat urine from rats 4, 5 and 6 after stomach intubation with 75 ug STX. The cumulative percent recovery of STX for rats 4, 5 and 6 were: 1.5, 1.3 and 1.1 res pectively. The data show that STX can be found as soon as 0.5 hrs after dosing although the initial amount at this early date is minimal,i.e.,4.2 ng. The greatest quantities are found at later collection times: 3, 6, 7 and 8 hrs after treatment. It is notable that 31 hrs after treatment, 327 ng of STX was detected. This is the largest amount of all collections.

The numerical data presented in Table 5 is also shown in Figures 14, 15 and 16 for the individual rats. A side-by-side comparison of STX found in all three rats is shown in Figure 17. In general, greatest yields are found at 3, 4, 6, 7, 8 and 31 hrs after treatment. There appears to be a fluctuation in the amounts of STX excreted. We did not monitor beyond 31 hours but the data suggest that a significant amount could be found beyond this time of collection. This indicates that using urine as a determinant of intoxication is a reasonable and appropriate method of detection of STX insult in rats and presumably in humans. Data obtained from other experiments suggest that STX can be found in body fluids at least 2 to 3 days after exposure.

The absolute amount of STX (expressed in nanograms) found in the urine of rat 5 is shown in Figure 14. The volume of urine collected at each collection time varied from .04 to 0.8 ml as it

was not possible to control water consumption and collection; these were the two independent variables. However, in general, the results suggest that for the first hour there is little excretion of the toxin but 2 to 31 hrs afterward (with the exception of 5 and 27 hrs), significant amounts are excreted. Similar results were found in the urine of rat 4 (figure 15), i.e., significant amounts of STX were found after 3 to 31 hrs (with the exception of hours 5 and 12). The amount of STX found in the urine of rat #6 (figure 16) varied slightly from rats 4 and 5 in that STX was found as soon as 0.5 hrs after treatment, the larger amounts being excreted at 3 hrs. Side-by-side comparison (Figure 17) of STX excreted from each rat indicates that at 3 and 4 hrs, one can expect a significant amount of STX to be excreted followed by 6 to 8 hrs, 25 and 31 hrs. The important finding in this experiment is that indeed STX is excreted in significant amounts after 3 hrs and up to 31 hrs and monitoring of urine is effective in rats, and by projection, in humans intoxicated with the toxin.

Confirmation by spectrometry of STX in rat urine: CF/FAB/MS and microcapillary columns (320 microns i.d. x 15 cm) were used to confirm the identity of STX in the urine samples. Resolution of STX standard (20 ng) injected in 0.5 ul is shown in Figure 18A (mass chromatogram of m/z+ 300). A gradient elution system was used as described in the methods section resulting in retention time of about 14 minutes. The full scan mass spectrum of the resolved standard is shown in figure 18B. The protonated molecular ion is found at m/z 300; fragments found at m/z+ 277 and 369 are due to the glycerol matrix. The fragment at m/z+ 387 is not identified

but can be found in both the standard and the urine analyses. It might be an adduct involving the pentafluoro- propionic acid matrix although this is only speculative.

The STX found in rat 2 (dosed by stomach intubation) urine was resolved as shown in the mass chromatogram of m/z+ 300 (Figure 19-A). The large amounts of salts found in the sample (due to oxidation reagents) detract from the chromatography shown with the standard in Figure 19A; however, STX was resolved and its spectrum recorded. Figure 19B shows the full scan FAB mass spectrum of STX found in the urine. The molecular ion found at m/z+ 300 is the major peak. Notable in this chromatographic resolution is the absence of m/z+ 301 found in the rat and human urine which normally interferes with STX analysis. The m/z+ 301 component is shown in Figure 20-A at retention time 2.2; shown with STX at 8.5 min. Although the amounts found in urine are large, it no longer interferes with STX detection. The m/z+ 301 component is resolved between 2 to 3 min. and is not present with STX found at about 8.5 min. The quantity is reduced considerably by microdialysis; the FAB mass spectrum of the component at m/z+ 301 is shown in Figure 20-B.

The presence of STX in spiked human urine after 23 hrs of microdialysis is shown in Figure 21-A. The chromatography is somewhat improved because of the absence of salts removed by dialysis. The FAB spectrum of STX found in the urine is shown in Figure 21-B. The protonated molecular ion at m/z+ 300 is the major peak and m/z+ 282 is present and adds to the confirmation. The interfering substance found at m/z+ 301 is not present in this preparation.

Microdialysis shows promise as an important step in obtaining a good clean mass spectrum as well as being helping in resolution of STX on the microcolumn.

There are at least two important and significant findings concerning urine excretion of STX: (i) it is possible to find STX in urine of the rat up to 8 hrs and blood up to 24 hrs after dosing (i.p.) with as little as 1.5 ug of STX per adult rat and 31 hrs by stomach intubation (ii) the kinetics of STX recovery suggests an initial excretion of the toxin into the urine, followed by release at hours 4 to 8. The latter is a bimodal excretion pattern suggesting an interaction within the rat. Presently we have no evidence for metabolism of STX by the female rat. This may be due in part to the lack of resolution of peroxidation products, formed or not resolved, or because peroxidation prevents discrimination between derivatives such as neosaxitoxin. An example of this is given by the report of Lawrence,et al.,(1991) where the N-1-hydroxylated toxins (neosaxitoxin, B-2, GTX-1 and C-3) do not form fluorescent compounds with hydrogen peroxide oxidation whereas they do with periodate oxidation. On the other hand, STX, B-1, GTX-2, GTX-3, C-1 and C-2 form highly fluorescent derivatives with both periodate and hydrogen peroxide.

Detection of STX in the urine of the rat treated with 75 ug STX was followed only up to 31 hrs; interpretation of the results indicate that detection could be extended at least up to 36 hrs. The latter is of assistance in intoxication of humans where urine may not be collected until 24 hrs after initial symptoms appear or correlations are made with ingestion of contaminated mussels.

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Table 1. The concentration of STX in the serum of rats dosed with 1.5 μg (i.p.) of STX per rat.

Time (hr.)*	Concentration of STX** (ng/ml)	Average (ng/ml)	Standard deviation ± Sd
0.2	10.10; 15.83	12.97	2.87
1.0	3.82; 4.34	4.08	0.26
2.0	3.47; 3.23	3.35	0.12
3.0	2.96; 1.96	2.46	0.5
4.0	2.43; 3.90	3.17	0.74
6.0	2.07; 1.96	2.02	0.06
8.0	2.42; 3.05	2.74	0.32
12.0	2.27; 2.17	2.22	0.05
24.0	1.89; 1.76	1.83	0.07
Control	0.00; 0.00	0.00	0.00

* = Time after dosing.

** = Concentration of STX found in individual rat.

Table 2. Concentration of (ng/ml) in rat urine at various time intervals after dosing (i.p.) of rats with 1 μg of saxitoxin on days 1 and 2 and concentration of STX found on day 3 and 4 μg dosing.

Time (day-hr.)	Concentration of saxitoxin in urine (ng/ml)				
	Rat 5	Rat 6	Rat 7	Average	± Sd
1-1	284.8	34.2	226.1	181.7	107.0
1-2	14.0	ND**	27.8	13.9	11.4
1-3	47.4	ND	45.9	31.1	22.0
1-4	78.0	5.3	139.0	74.1	33.4
1-6	61.8	ND**	64.1	42.0	29.7
1-8	85.4	7.6	132.6	75.2	37.9
2-1	12.1	32.0	57.5	33.9	18.6
2-2	11.3	23.1	37.7	24.0	10.8
2-3	16.2	32.0	59.0	35.7	17.7
2-4	48.2	86.0	154.9	96.4	44.2
2-6	85.1	13.7	N/A	49.4	35.7
2-8	45.8	83.9	76.9	68.9	16.6
3-0.1*	N/A	665.0	596.5	630.8	34.3

* = Rat dosed with 4 μg STX at 0900 on day 3.

** = Not detected.

Table 3. Total saxitoxin recovered in rat urine at various time intervals after dosing (i.p.) of rats with 1 μg of saxitoxin on days 1 and 2 and total STX found on day 3 after 4 μg dosing.

Time (day-hr.)	Total of Saxitoxin (ng)				
	Rat 5	Rat 6	Rat 7	Average	± Sd
1-1	310.5	13.4	108.5	144.1	123.9
1-2	2.8	ND**	6.7	3.2	2.7
1-3	13.3	ND**	6.9	6.7	5.4
1-4	60.1	0.4	95.9	52.1	39.4
1-6	26.0	ND**	25.6	17.2	12.2
1-8	24.8	2.6	10.6	12.7	9.2
2-1	3.6	8.0	11.5	7.7	3.2
2-2	2.8	6.7	30.9	13.4	12.4
2-3	2.8	9.0	11.2	7.6	3.6
2-4	11.1	17.2	83.6	37.3	32.9
2-6	17.9	3.9	N/A	10.9	7.0
2-8	4.6	8.4	29.2	14.1	10.8
3-0.1*	N/A	20.0	41.8	30.9	10.9

* = Rat dosed with 4 μg STX at 0900 on day 3.

** = Not detected.

Table 4. Urine volume, concentration (ng/ml), and absolute amount of STX in urine of rats 1, 2 and 3 collected after being dosed (stomach intubation) with 75 μg of STX.

Time (hr)	Volume (ml)	Concentration of STX (ng/ml)	Absolute amount of STX (ng)
		Test-1 (rat 1)	
0.5	0.41	52.8	21.6
1.0	0.25	103.6	26.3
2.0	0.34	278.5	94.1
3.0	0.38	200.6	75.8
4.0	1.29	52.3	67.5
5.0	0.06	171.7	9.6
6.0	0.49	47.8	23.4
7.0	0.40	60.5	24.4
8.0	0.43	88.5	37.8
12.0	1.67	26.8	44.8
25.0	0.91	134.8	122.7
27.0	0.39	416.4	162.2
31.0	0.25	44.9	11.1
		Test-2 (rat 2)	
0.5	0.28	25.5	7.2
1.0	0.18	89.8	15.9
2.0	0.73	22.1	16.1
3.0	0.29	69.0	20.0
4.0	0.96	2695.4	2587.2
5.0	1.50	1387.9	2080.5
6.0	0.29	405.2	117.5
7.0	1.09	1530.6	1668.4
8.0	0.86	606.9	521.9
12.0	0.10	291.5	29.2
25.0	0.57	530.8	302.6
27.0	0.60	217.5	130.5
31.0	0.61	106.4	64.9
		Test-3 (rat 3)	
0.5	0.47	121.1	56.9
1.0	0.04	5703.5	199.6
2.0	0.30	162.3	48.7
3.0	0.60	127.5	76.5
4.0	0.55	119.9	65.9
5.0	0.40	136.7	54.7
6.0	0.04	913.2	36.5

Table 4. (Continued)

Time (hr)	Volume (ml)	Concentration of STX (ng/ml)	Absolute amount of STX (ng)
7.0	0.36	40.4	14.5
8.0	0.35	78.7	27.5
12.0	0.66	43.7	28.8
25.0	0.76	48.4	36.8
27.0	0.60	59.1	35.5
31.0	0.85	61.8	52.5

Table 5. Urine volume, concentration (ng/ml), and absolute amount of STX in urine of rats 4, 5 and 6 collected after being dosed (stomach intubation) with 75 μg of STX.

Time (hr)	Volume (ml)	Concentration of STX (ng/ml)	Amount of STX (ng) absolute	Amount of STX (ng) per injection*
		Test-4 (rat 4)		
0.5	0.10	41.8	4.2	0.21
1.0	0.03	306.0	9.2	0.46
2.0	0.03	169.9	5.1	0.25
3.0	0.60	290.9	174.5	1.45
4.0	0.25	130.2	32.6	0.65
5.0	0.08	105.4	8.4	0.42
6.0	0.56	285.8	160.1	1.43
7.0	0.44	415.4	182.8	2.08
8.0	0.44	377.8	166.2	1.89
12.0	0.05	109.6	5.5	0.27
25.0	1.01	36.1	39.7	0.18
27.0	0.13	240.7	31.3	1.20
31.0	1.02	321.3	327.7	1.61
		Test-5 (rat 5)		
0.5	0.04	186.1	7.4	0.37
1.0	N/A	N/A	N/A	N/A
2.0	0.66	180.9	119.4	0.90
3.0	0.53	263.4	139.6	1.32
4.0	0.57	332.1	189.3	1.66
5.0	N/A	N/A	N/A	N/A
6.0	0.31	376.3	116.7	1.88
7.0	0.75	278.6	208.9	1.39
8.0	0.21	189.6	39.8	0.95
12.0	0.31	54.9	17.0	0.27
25.0	0.80	67.2	53.8	0.34
27.0	0.09	117.2	10.6	0.53
31.0	0.63	157.7	99.4	0.79
		Test-6 (rat 6)		
0.5	0.18	125.4	22.6	0.63
1.0	0.07	1093.7	76.6	3.83
2.0	0.06	199.9	12.0	0.60
3.0	0.36	435.0	156.6	2.18
4.0	0.45	184.5	83.0	0.92
5.0	0.21	303.2	63.7	1.52
6.0	0.02	288.4	5.8	0.29

Table 5. (Continued)

Time (hr)	Volume (ml)	Concentration of STX (ng/ml)	Amount of STX (ng)	
			absolute	per injection*
7.0	0.32	624.6	199.9	3.12
8.0	N/A	N/A	N/A	N/A
12.0	0.14	113.6	15.9	0.57
25.0	0.14	35.0	4.9	0.18
27.0	0.21	687.6	144.4	3.44
31.0	0.75	57.8	43.4	0.29

* = Amount of STX in the HPLC injection volume.

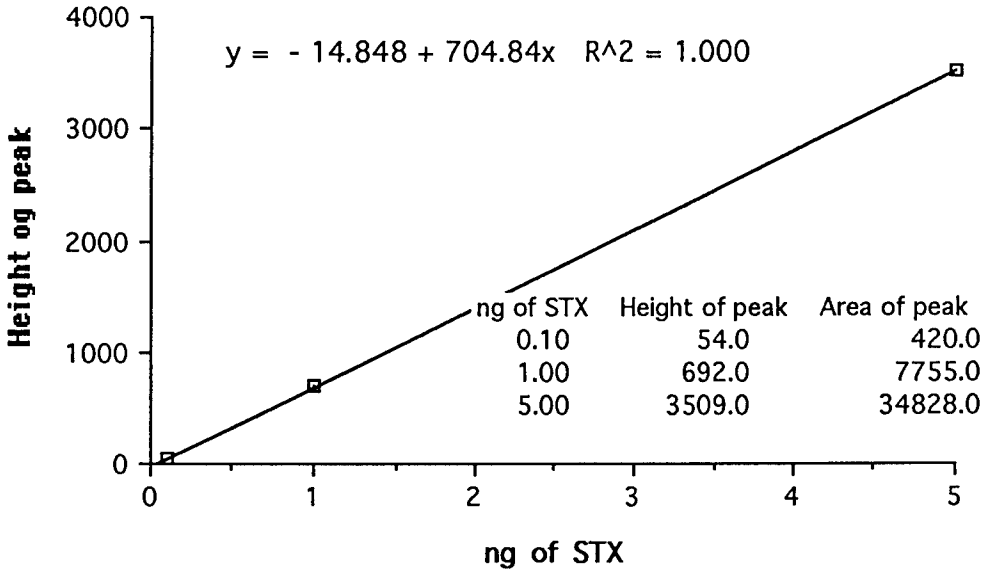


Figure 1. Standard curve, based on peak height of saxitoxin, after peroxide oxidation, resolution on a C18 column by HPLC and detection by fluorescence. The limit of detection is 100 pg. The actual peak height and area values are in the inset.

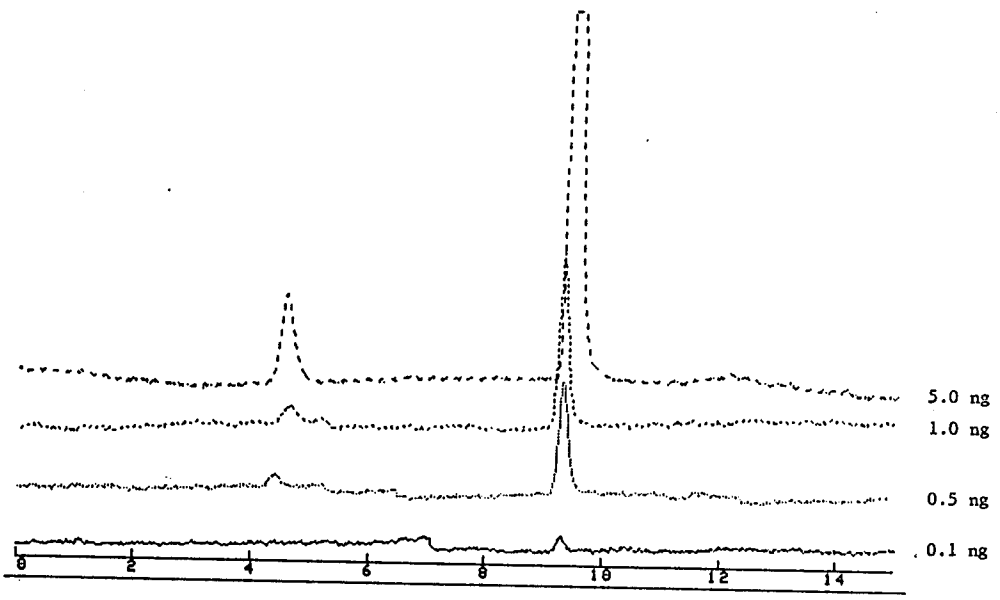


Figure 2. Detection of STX after oxidation and resolution by HPLC. The recorder responses are superimposed (overlay) in a linear fashion and represent a total of 5, 1, 0.5, and 0.1 ng of STX. Note the unknown component at retention time 4.5. The ordinate is shown in relative units,i.e., 0 to 100%.

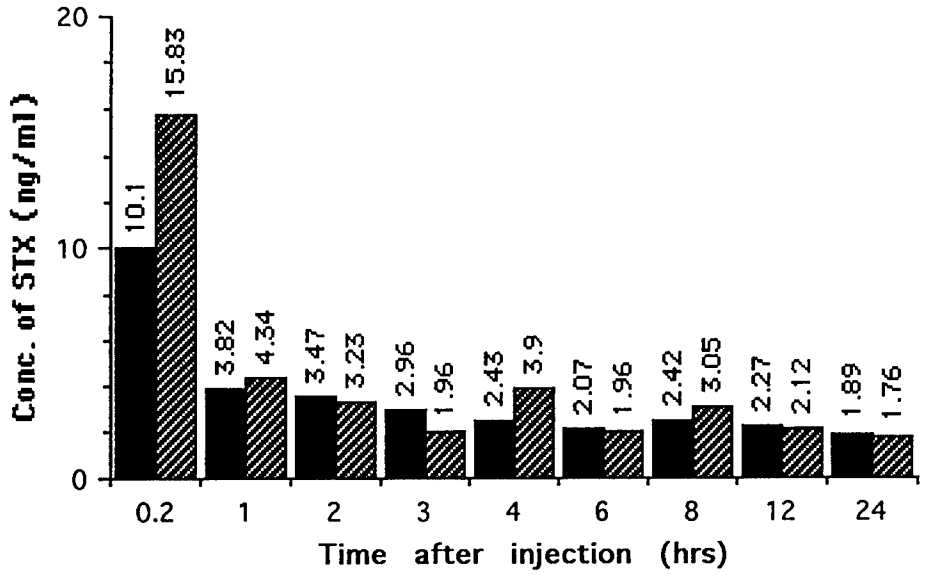


Figure 3. Concentration (ng/ml) of STX found in the blood serum of rats treated with STX (1.5 ug total dose) by intraperitoneal injection. The solid and hatched bars represent rat pairs sacrificed at each time increment. The values above each bar is the concentration of STX in the serum of each rat.

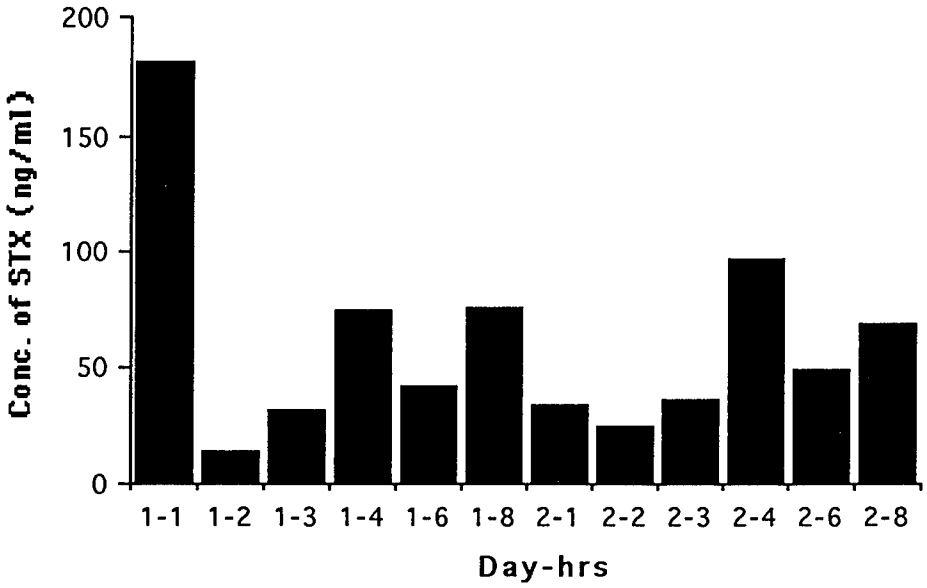


Figure 4. Average concentration (ng/ml) of STX found in the urine of three rats (5, 6 and 7) dosed (i.p) with 1 ug STX per 300-350 g rat. Each rat represents a separate experiment

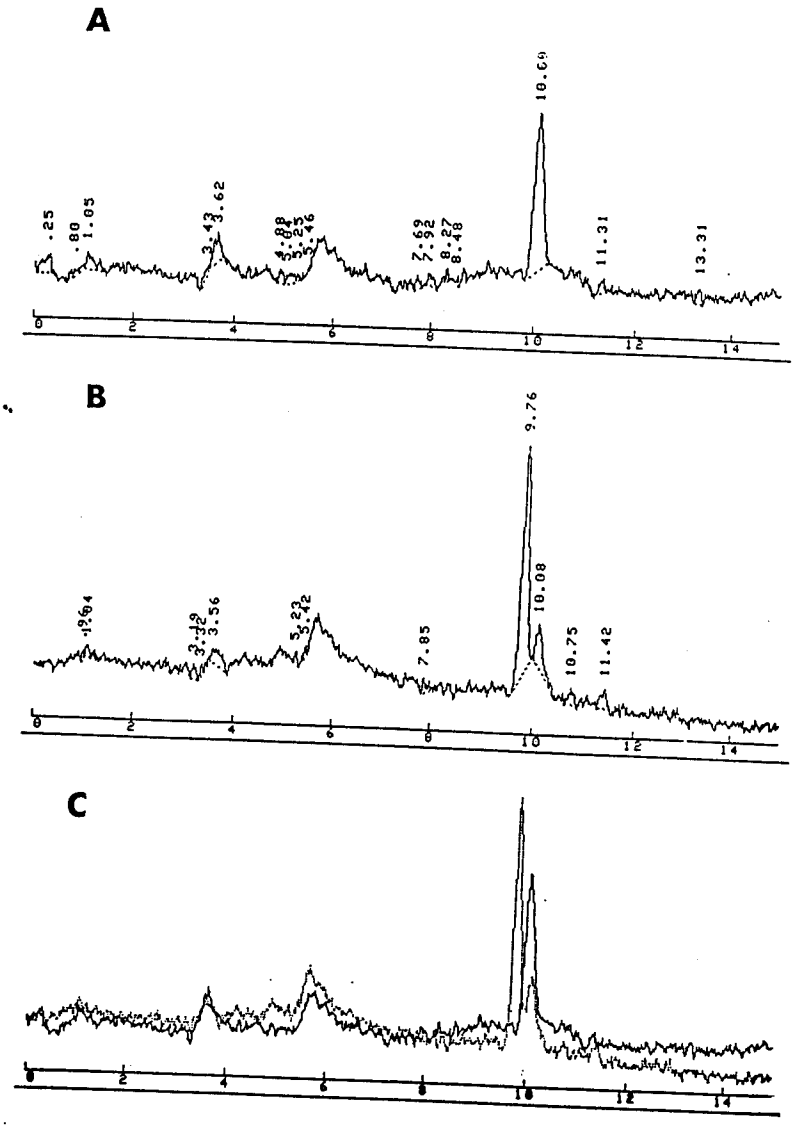


Figure 5-ABC.(A) Oxidation product (retention time 10.0) found in the urine of control rats (not treated with STX) when analyzed by HPLC. (B) Oxidation product of STX (retention time 9.7) found in urine of rat 5 collected in the 8th hour of day 1 of treatment. The concentration of STX was 85.4 ng/ml. (C) Overlay of A and B show complete resolution of STX. The ordinate represents percent relative intensity (0-100%).

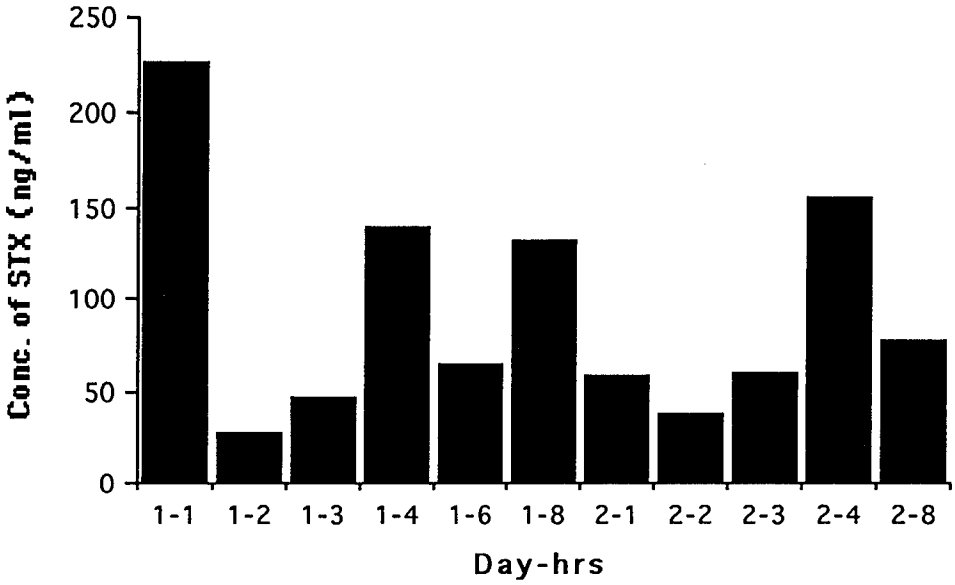


Figure 6. Concentration of STX (ng/ml) in urine of rat 7 collected at 1, 2, 3, 4, 6 and 8 hrs after dosing (i.p.) with 1 ug STX on days 1 and 2.

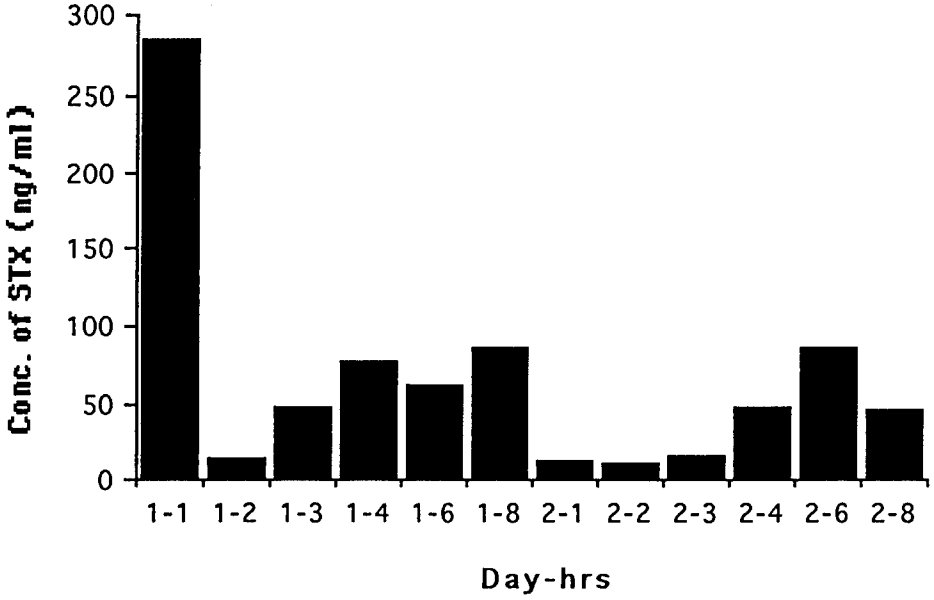


Figure 7. Concentration of STX (ng/ml) in urine of rat 5 collected at 1, 2, 3, 4, 6 & 8 hrs after dosing with 1 ug STX on days 1 and 2. Note the similarity in pattern to rat 7.

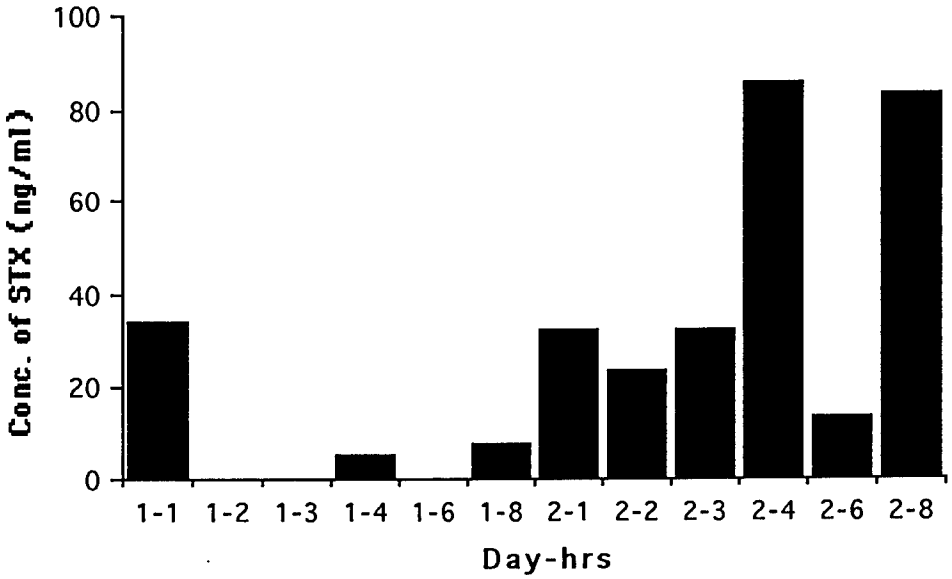


Figure 8. Concentration of STX (ng/ml) in urine of rat 6 collected at 1, 2, 3, 4, 6 and 8 hrs after dosing (i.p.) with 1 ug STX on days 1 and 2. Note the large amount of STX found in the urine 4 and 8 hrs after treatment on day 2.

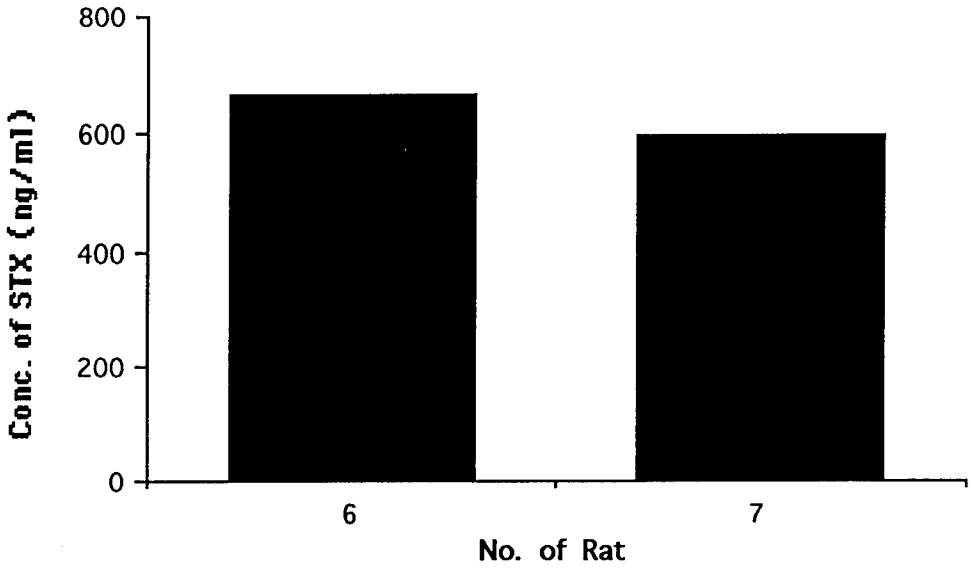
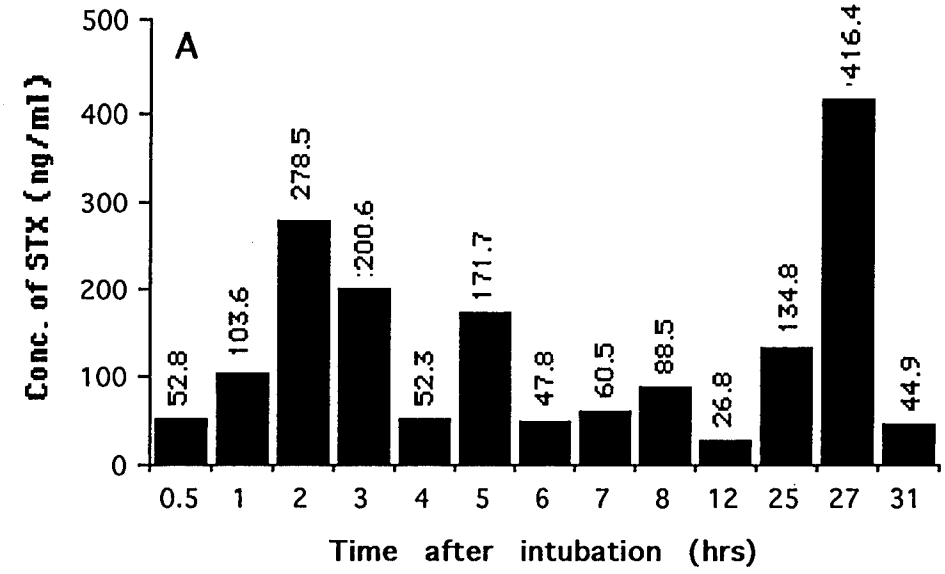


Figure 9. Concentration (ng/ml) of STX found in urine of rats 6 and 7 days after their initial insult (i.p.) of 1 ug STX followed by a dose (i.p.) of 4 ug given on day 3. The animals died after 3 min and the urine was collected by 10 min.



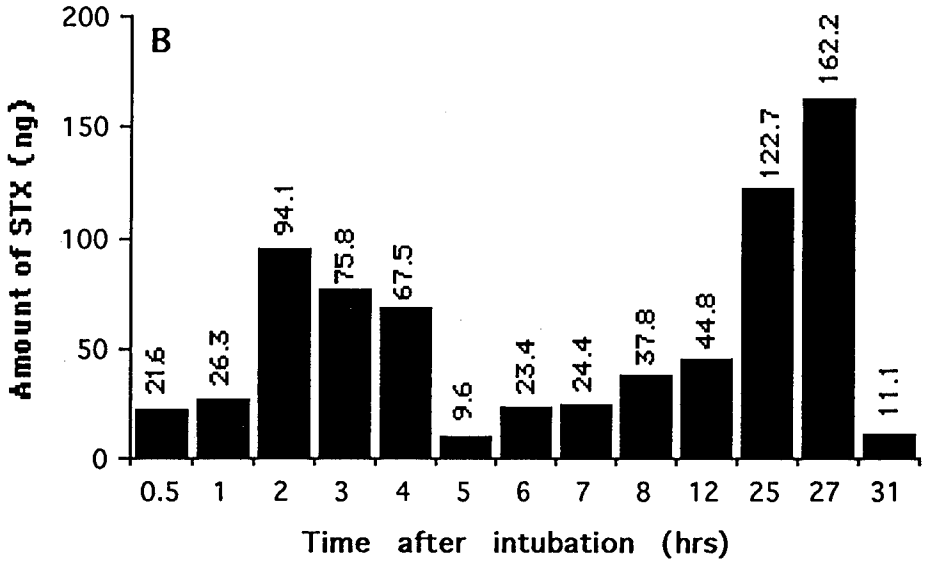


Figure 10A-B. Concentration of STX in the urine of rat 1 after stomach intubation with 75 ug STX. The value above each bar represent the exact STX concentration at each collection time. Absolute values have been normalized to ng/ml. (B) Absolute amount in nanograms of STX found in the urine of rat 1.

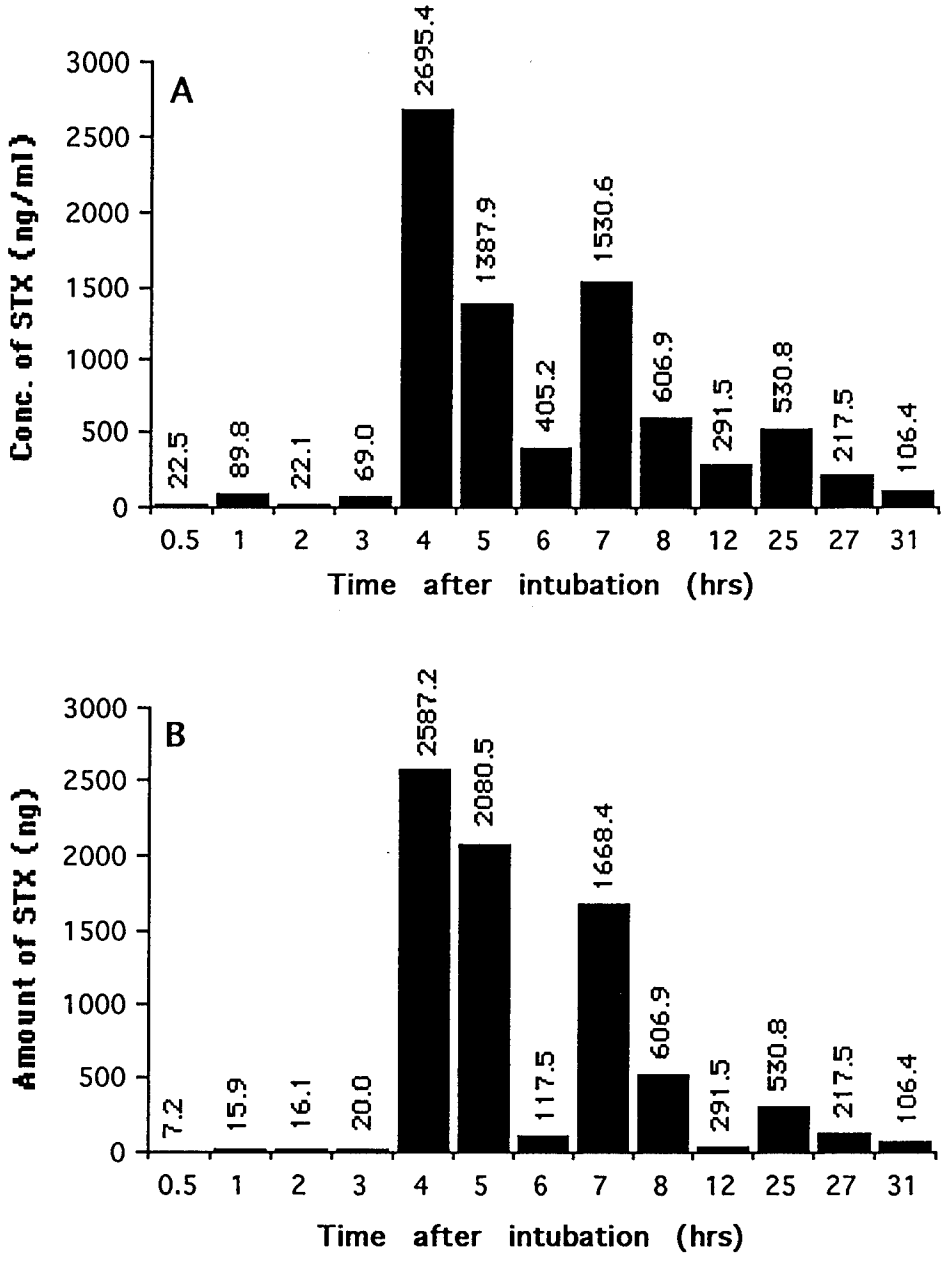


Figure 11A-B. Concentration of STX in the urine of rat 2 after stomach intubation with 75 ug STX. The value above each bar represents the exact STX concentration at each collection time. (B) Absolute amount in nanograms of STX found in the urine of rat 2.

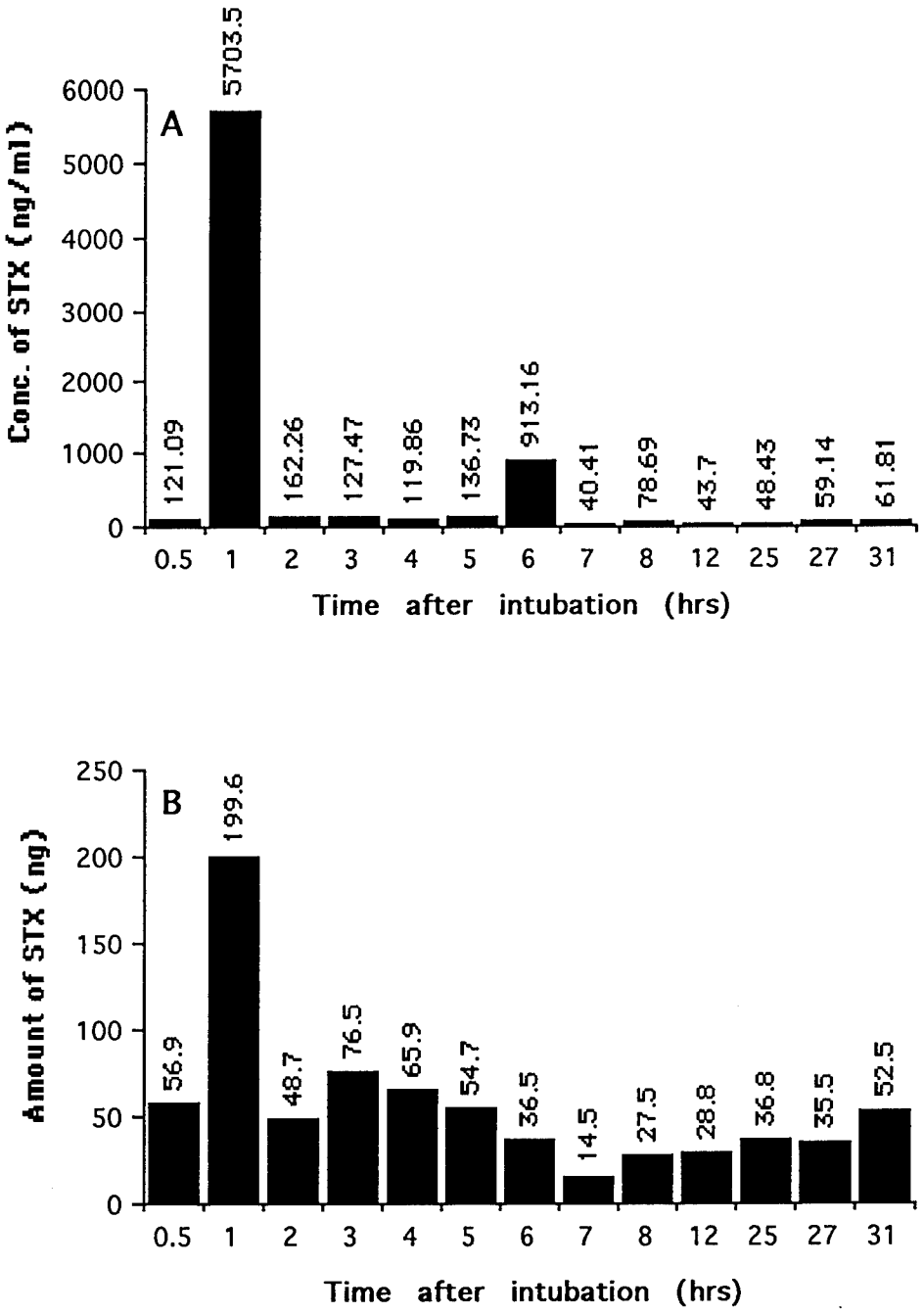


Figure 12A-B. Concentration of STX in the urine of rat 3 after stomach intubation with 75 ug STX. The value above each bar represents the exact STX concentration at each collection time. (B) Absolute amount in nanograms of STX found in the urine of rat 3.

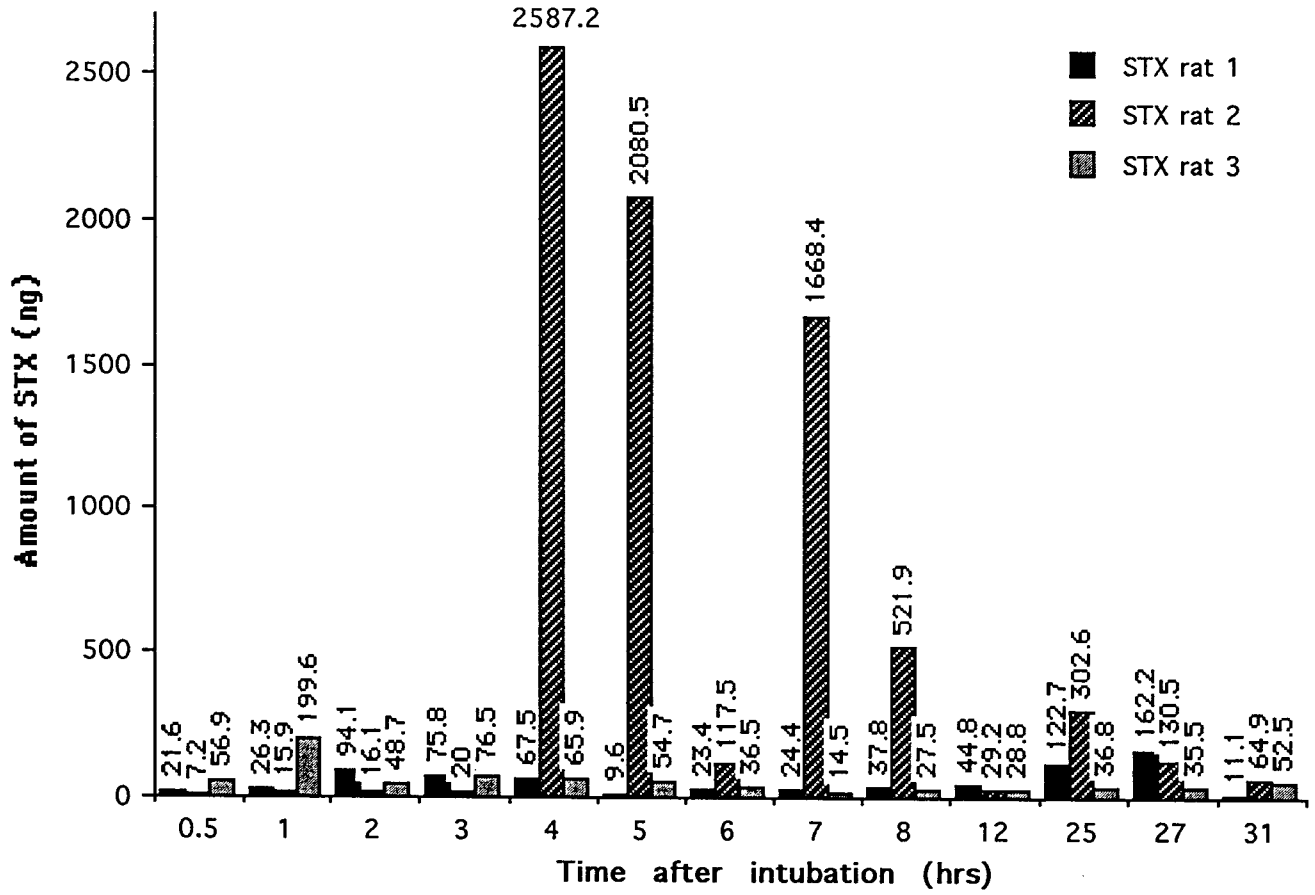


Figure 13. Comparison of STX found in the urine of rats 1, 2 and 3 dosed with 75 ug STX by stomach intubation. This figure serves as a summary of the data on all 3 rats.

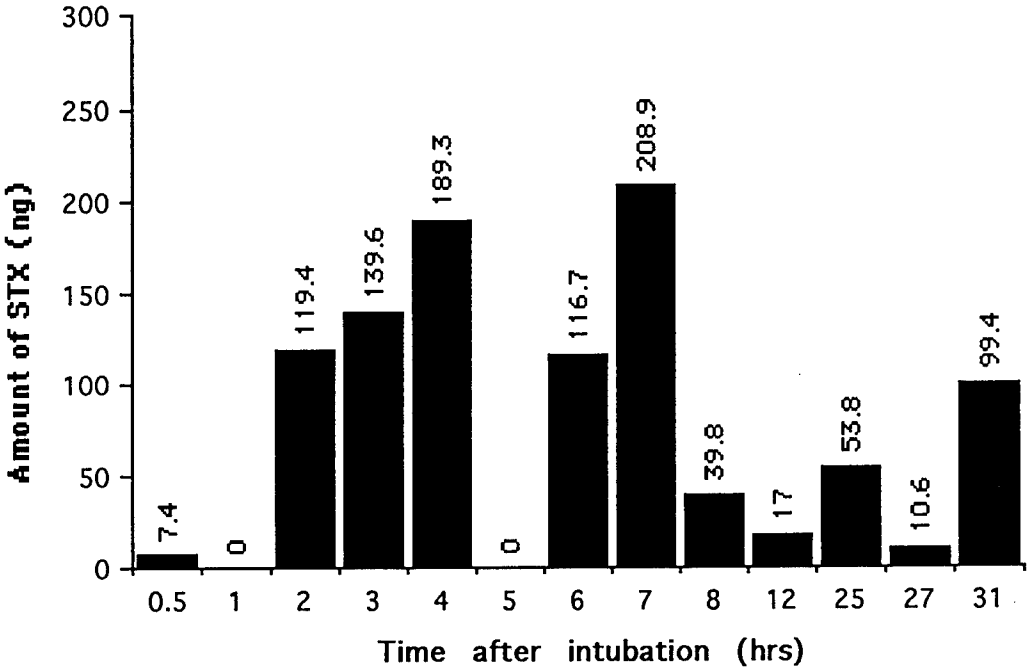


Figure 14. Absolute amount of STX in urine of rat 5 after being dosed (stomach intubation) with 75 ug STX.

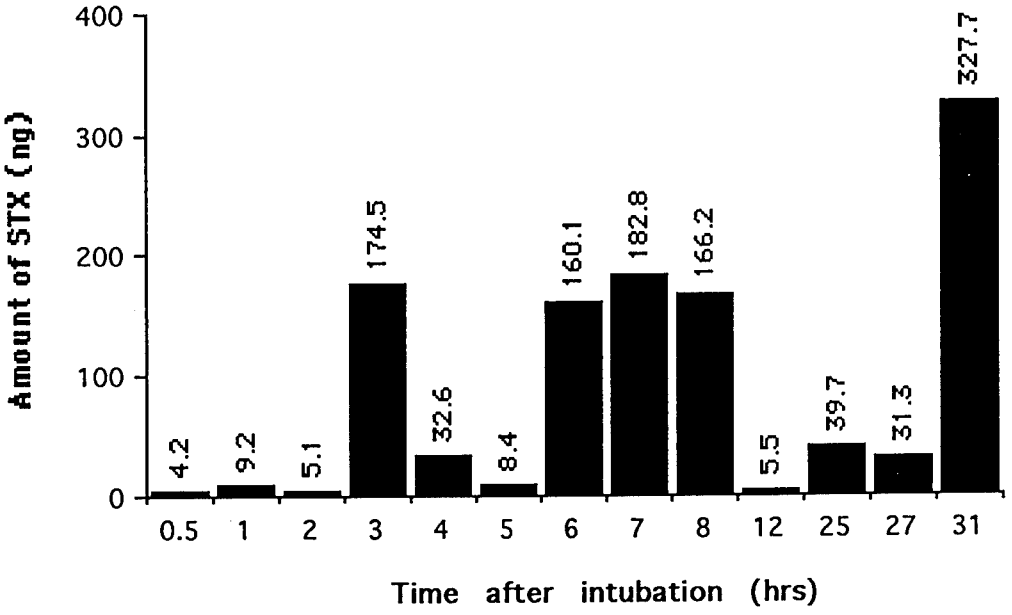


Figure 15. Absolute amount of STX in urine of rat 4 after being dosed (stomach intubation) with 75 ug STX.

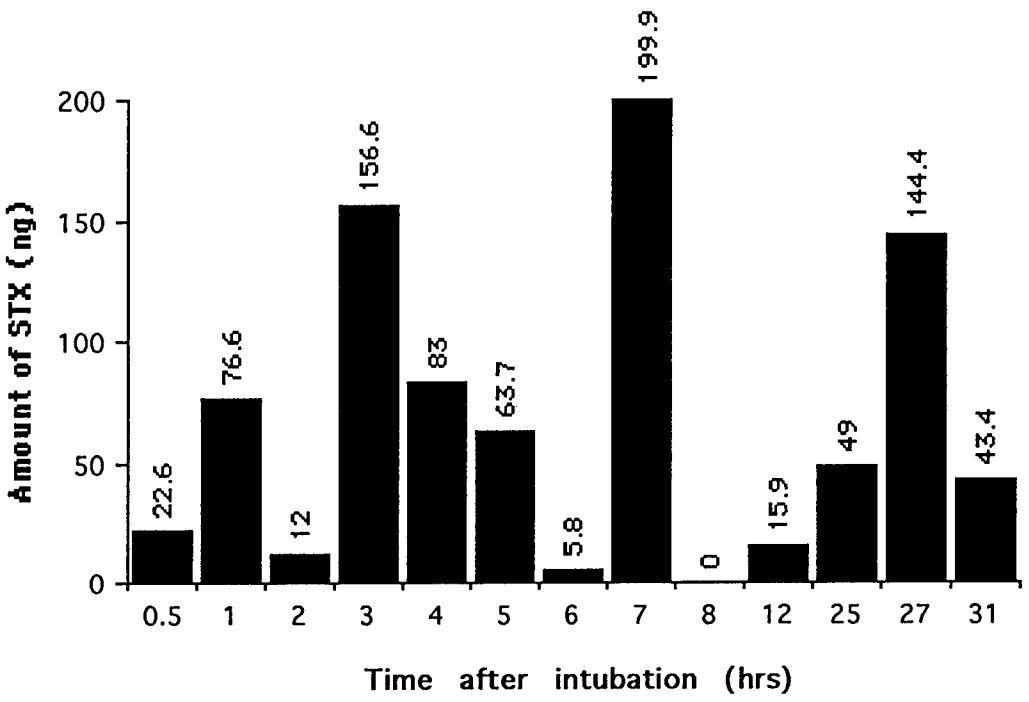


Figure 16. Absolute amount of STX in urine of rat 6 after being dosed (stomach intubation) with 75 ug STX.

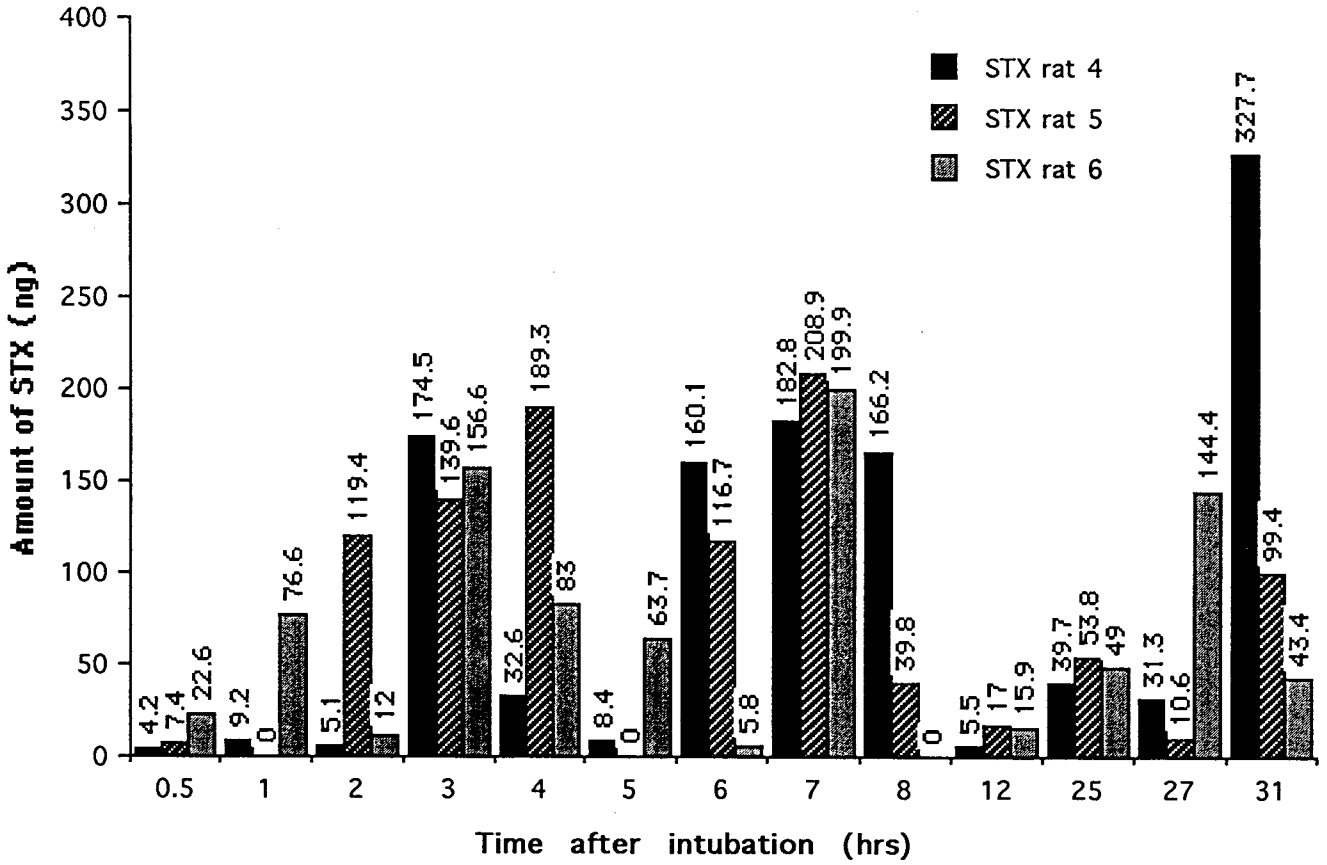


Figure 17. Comparison of the amount of STX in the urine of rats 4, 5 and 6 after being dosed by stomach intubation with 75 ug STX.

PART II.

USE OF EMPORE MEMBRANES FOR PURIFICATION OF SAXITOXIN IN HUMAN AND RAT URINE FOR ANALYSIS BY HPLC AND CF/FAB/MS

ABSTRACT

An analytical method for the detection of saxitoxin (STX) in human and rat urine samples was developed using high performance liquid chromatography (HPLC) and continuous flow fast atom bombardment mass spectrometry (CF/FAB/MS). The urine samples were passed directly through an EMPORE particle-loaded PTFE membrane containing an experimental weak cation exchange resin and eluted with 10% acetic acid. Analysis was made either by HPLC or CF/FAB/MS. The limit of detection by HPLC was 4 ng/ml (4 ppb) in human urine and 220 ng/ml (220 ppb) in rat urine. The percent recovery (by HPLC) for concentrations 220, 550 and 1100 ppb of STX contained in 100 ul of rat urine was 102, 93 and 81% respectively. The recovery of STX concentrations of 4.3, 11.5 and 23 ppb (contained in 5 ml human urine) were 78, 82 and 68 % respectively. The percent recoveries by CF/FAB/MS, using microcapillary columns, for STX concentrations of 25, 50 & 100 ppb in human urine were 103, 97 and 97% respectively. The limit of detection in human urine by CF/FAB/MS is 25 ppb.

INTRODUCTION

Various analytical methods have been devised including high performance liquid chromatography (Janecek et al. 1993) , capillary zone electrophoresis (Thibault et al. 1991), continuous flow fast

atom bombardment (Mirocha et al. 19920, and ion spray mass spectrometry (Quilliam et al. 1989). Perhaps the most useful and inexpensive is the HPLC method described by Lawrence et al. 1991 , where STX was oxidized with hydrogen peroxide prior to separation on the HPLC column and detection by fluorescence. Purification of STX from various biological substrates is critical for analysis.

The aim of this work was to develop a novel method of saxitoxin (STX) isolation and purification using thin Empore particle-loaded membranes (disks) for sample preparation (instead of traditional packed columns), followed by analysis using high pressure liquid chromatography (HPLC) with fluorescence detection and continuous flow fast atom bombardment mass spectrometry (CF-FAB-MS).

EXPERIMENTAL

Chemicals: Acetic acid, acetonitrile, methanol and water of HPLC grade were purchased from EM Science Ind. Inc.(Gibbstown, NJ, USA) Ammonium formate, analytical grade, was purchased from Aldrich Chemical Co. Inc. (Milwaukee, WI, USA). Hydrogen peroxide (30% solution) was purchased from Fisher Scientific (Fair Lawn, NJ, USA). Saxitoxin standard was obtained from Dr. Harry Hines, Fort Detrick (Frederick , MD, USA). Empore membranes (0.5 mm thickness) were obtained from 3M Company (St. Paul, MN. USA) and contained 8 um (avg) carboxylated poly(styrene divinylbenzene) resin particles tightly loaded within a polytetrafluoroethylene (PTFE) matrix (90:10.w/w). Membranes were mounted into standard 6cc polypropylene syringe barrels to yield an effective membrane diameter of 11 mm. The carboxylated particles were prepared in two experimental

formats, with capacities of either 0.9 mEq/g (mid capacity) or 1.5 mEq/g (high capacity). Sodium phosphate (Na2HPO4/NaH2PO4) buffer (pH=8) was prepared of salts obtained from Mallincrodt Inc. (Paris, KY, USA).

Equipment: HPLC SLC-6A (Shimadzu Co. Kyoto, Japan) with C18 Nova-Pak reverse phase column 75 mm x 3.9 mm i.d. (Waters, Millipore Corporation, Milford, MA, USA) and Shimadzu RF 0530 fluorescence HPLC Monitor detector (wavelength: Ex=350, Em=410 nm) were used. A vacuum manifold system (Supelco, Bellefonte, PA, USA) was used for solid phase extraction and cleanup procedure. A micro-dialyzer (Spectra/Por, Medical Industries, Inc. Spectrum, Houston, TX, USA) with a cellulose ester membrane (molecular weight cutoff 0:100) was used to dialyze saxitoxin eluate off the PTFE membrane to remove salts. The mass spectrometer used was a VG7070EQ equipped with CF/FAB. The syringe pump used in solvent control of the HPLC was an ABI-140B, Applied Biosystems Inc.

Sample Preparation: Small (100 ul) volumes of rat urine samples, spiked with saxitoxin, were prepared in the following manner: known amounts (22, 55 and 111 ng) of saxitoxin standard in aqueous solution was added to a 1 dram vial and after evaporation of water, 100 ul of rat urine was added. Sample was loaded on PTFE membrane in 2 ml sodium phosphate (Na2HPO4/NaH2PO4) buffer (pH=8). Larger (5 ml) volumes of human urine samples, spiked with saxitoxin, were prepared as follows: human urine was adjusted to pH = 8 with 1 N NaOH; known amounts (14.5, 21.7, 57.8 and 115.6 ng) of saxitoxin standard in aqueous solution were added to 4 dram vials and after

evaporation of water, 5 ml of human urine (pH=8) was added. The sample was then loaded on the PTFE membrane.

Purification of urine samples on PTFE membranes: PTFE membranes of mid or high capacity were conditioned with 2 ml of isopropanol followed by 1 ml of methanol and 1 ml of sodium phosphate (Na_2HPO_4/NaH_2PO_4) buffer (pH=8) in preparation of rat urine samples. Human urine samples were prepared as above except that 1 ml of water was used instead of buffer. Urine samples (see sample preparation) were loaded on the column after conditioning after which the column was washed with 2 ml of water followed by 1 ml of methanol. Saxitoxin was eluted from the column with four 0.5 ml portions of acetic acid (10%) and the eluate was dried under a stream of air at ambient temperature. The same procedure was used when the rat urine samples were purified on CBA columns, but the saxitoxin was eluted in this case with 2 ml of acetic acid (10%).

Microdialysis: After elution of the sample from the PTFE membrane, some of the fractions were dialyzed to remove salts. A micro dialyzer (Spectra/Por, Medical Industries, Inc., Spectrum) with a cellulose ester membrane (molecular weight cutoff 0:100) was used; the latter allowed the phosphate salts to pass through but retained most of the saxitoxin and other materials with molecular weight greater than 100. About 0.5 ml of the eluate was loaded into 0.6 ml dialysis wells and dialysis was performed for 20 hrs with a flow rate of 5 ml/min of distilled water. Dialyzed samples were evaporated to dryness under an air stream at ambient temperature.

Oxidation of STX: The column eluate after dialysis was dissolved in 100 ul of water after which 25 ul of hydrogen peroxide (10%) and 250 ul of 1 N NaOH were added. After 5 min of oxidation reaction, 25 ul of concentrated acetic acid was added to quench the reaction.

HPLC analysis of saxitoxin: Products of the oxidation reaction were injected into an HPLC column via a 20 ul loop. The following solvents and conditions were used to elute saxitoxin: solvent A - 0.1 M ammonium formate in water; solvent B-20% of acetonitrile in solvent A; time program 0.1 min of A at 1 ml/min with solvent B at 0 ml/min; 14 min of A at 0.7 ml/min and B at 0.3 ml/min; 15 min of A at 1 ml/min and B at 0 ml/min. Fluorescence detector (wavelength: Ex=350, Em=410 nm) was used.

Preparation of the standard curve: The standard curve was prepared for each experiment separately and the level of saxitoxin was chosen according to the level of the toxin expected in the analytical sample,i.e.,the concentrations chosen (prepared in four replicates each) bracketed those found in the sample. Known amounts were diluted to a volume of 100 ul, and an oxidation reaction followed by HPLC analysis was performed as described.

RESULTS and DISCUSSION

Analysis of Rat Urine by HPLC: The Empore particle-loaded membrane is comprised of densely packed (90 % by weight) sorbent particles which are immobilized within a stable, inert matrix of PTFE fibrils. The thin (0.5 mm) membrane was loaded with experimental poly(styrene divinylbenzene) carboxylated particles (avg 8 um) for

this study and used in a diameter of 11 mm in a disposable cartridge. In this format, solid phase extraction has been transformed from large particles (40-60 um avg) packed in a column into a thin membrane system. Uniform particle packing and the absence of voids, or walls, in the particle bed eliminates channeling, and the small bed volume reduces the amount of solvent required to elute the adsorbed analyte. The high and low capacity PTFE membranes are efficient for extraction and purification of 5 ml as well as 100 microliter volumes of urine as used in human and rat urines respectively. Rat urine volumes are small because of availability and are usually more concentrated and viscous than human urine.

The saxitoxin eluting off the PTFE membrane was found in the first 0.5 ml fraction; this is ideal for recovery experiments because the eluting volume is minimal and very little needs to be done to concentrate the sample. A standard curve (linear relationship based on peak height, ranging between 0.5 and 5.5 ng STX per injection) used for analysis of rat urine is shown in Figure 2. The data points are an average of 4 replicates.

Micro dialysis was used to reduce the amount of impurities accompanying the metabolite isolated from rat urine. A comparison was made between separation by a PTFE membrane without micro dialysis (Table 1) and with micro dialysis (Table 2) in order to deter mine whether the desalting step is necessary. The percent recovery as determined by HPLC, for concentrations of STX at 0.22, 0.55 and 1.11 ppm in rat urine without micro dialysis was 102, 93 and 81

respectively. These same concentrations with micro dialysis yielded respectively 98, 89 and 79 percent recoveries. Micro dialysis does not appear to be a critical step in the analysis of STX by HPLC.

Analysis Human Urine by HPLC: Five ml volumes of human urine were amended with STX at concentrations of 23, 12, and 4 ppb (ng/ml); the percent recovery was 78, 82, and 68 respectively (Table 3). The average is based on three separate experiments. An HPLC chromatogram of STX extracted from human urine containing 23.1 ng/ml (23.1 ppb) is shown in Figure 3.

Analysis Human Urine by CF/FAB/MS: A recovery experiment of STX in human urine was attempted with PTFE membranes and urine spiked at 25, 50 and 100 ppb. The same procedure of analysis was used as with rat urine. Three replicates of each was used in the determination. The average percent recovery found for the 25 ppb concentration was 102.6 (102.7, 102.7 and 102.5) and 97 (99, 94.5 and 97.5) for 50 ppb and 97 (95.7, 94.7 and 97) for the 100 ppb concentration. One-half microliter volumes were used for each injection with a total amount injected of 1.56ng, 3.12ng and 6.25 ng respectively. The calculations for the amount injected are shown below: 5 ml urine was concentrated and taken up in 40 ul of solvent and 0.5 ul was injected into the instrument.

25 ppb=25 ng/ml X 5 ml= 125 ng/40 ul =3.12 ng/2= 1.56 ng injected

50 ppb=50 ng/ml X 5 ml= 250 ng/40 ul = 6.25/2= 3.12 ng injected

100 ppb=100 ng/ml X 5 ml=500 ng/40 ul =12.5/2= 6.25 ng injected

The percent recovery was based on a standard curve (Figure 4) made from known concentrations of STX added to a human urine matrix ,i.e., control urine was purified on the PTFE membrane, eluted and then used as the medium for added STX. The total injected is well within the range of the standard curve.

The mass chromatogram of m/z+ 300 (protonated molecular ion) is shown in Figure 5. The high capacity PTFE membrane gave superior enhancement of cleanup so that a reasonable selected ion chromatogram was obtained with superior sensitivity. The area count for the 50 ppb sample was 34,249.

The analyses indicate that rat urine when analyzed by HPLC using the oxidation product as a determinant, 220 ppb of STX can be readily detected. When human urine spiked with STX is analyzed by HPLC, 4 ppb STX is the limit of detection. Analyses by CF/FAB/MS have a limit of detection at 25 ppb. The two methods should be used in concert,i.e., HPLC for detection and or screening and CF/FAB/MS for verification. The determinant ions used for STX confirmation in the mass spectrometer are found at m/z+ 300 and 282.

Table 1. Percent recovery of saxitoxin analyzed by HPLC after addition to 0.1 ml of rat urine ranging in concentration between 0.22 and 1.11 ug/ml. Sample purification was done on a high capacity (1.5 mEq/g) PTFE membrane. Each average is based on three separate experiments.

Amount of saxitoxin ug/ml (ppm)		Recovery	Average	St. Dev.
added	detected	(%)	(%)	(%)
	0.21	97.2		
0.22	0.21	98.5	101.7	6.8
	0.24	109.5		
	0.51	92.1		
0.55	0.49	88.8	92.9	4.5
	0.54	97.7		
	0.91	81.7		
1.11	0.86	77.3	81.0	3.4
	0.93	84.0		

Table 2. Percent recovery of saxitoxin analyzed by HPLC after addition to 0.1 ml rat urine (concentration ranged between 0.22 to 1.11 ug/ml) and sample purification on a high capacity (1.5 mEq/g) PTFE membrane followed by microdialysis. Each average is based on three separate experiments.

Amount of saxitoxin ug/ml (ppm)		Recovery	Average	St. Dev.
added	detected	(%)	(%)	(%)
	0.21	94.4		
0.22	0.21	96.0	97.7	4.4
	0.23	102.7		
	0.49	88.9		
0.55	0.47	86.3	88.7	2.3
	0.50	90.9		
	0.89	80.3		
1.11	0.84	75.3	79.3	3.6
	0.91	82.4		

Table 3. Percent recovery of saxitoxin analyzed by HPLC after addition to 5 ml human urine in concentrations ranging between 2.89 23.12 ng/ml (ppb) and sample purification on a high capacity (1.5 mEq/g) PTFE membrane. Each average is based on three separate experiments.

Amount of saxitoxin ng/ml (ppb)		Recovery	Average	St. Dev.
added	detected	(%)	(%)	(%)
	19.63	84.9		
23.12	17.52	75.8	77.6	6.6
	16.65	72.0		
	9.35	80.9		
11.56	9.94	86.0	81.9	3.7
	9.10	78.7		
	3.09	71.2		
4.34	2.89	66.5	67.9	2.9
	2.86	66.0		

Table 4. Percent recovery of saxitoxin from human urine after extraction and cleanup on a high capacity (1.5 mEq/g) PTFE membrane and analysis by continuous flow (microcapillary column) FAB mass spectrometry. Saxitoxin was added to 5 ml urine in concentrations of 25, 50 and 100 ng/ml (ppb).

Amount of Saxitoxin ng/ml (ppb)		Recovery	Average	Standard Deviation
added	detected	(%)	(%)	(%)
	25.7	102.7		
25.0	25.7	102.7	102.6	0.1
	25.6	102.5		
	49.5	99.0		
50.0	47.3	94.5	97.0	2.3
	48.7	97.5		
100.0	95.7	95.7		
	94.7	94.7	97.0	1.9
	97.0	97.0		

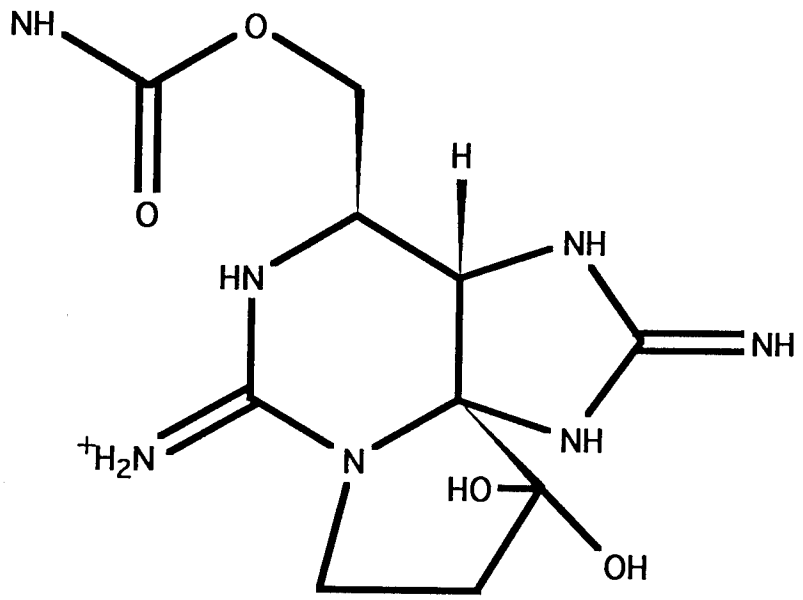


Figure 1. The chemical structure of saxitoxin.

	STX amount	Peak height
1	0.6	75
2	1.1	242
3	2.8	822
4	5.5	1753

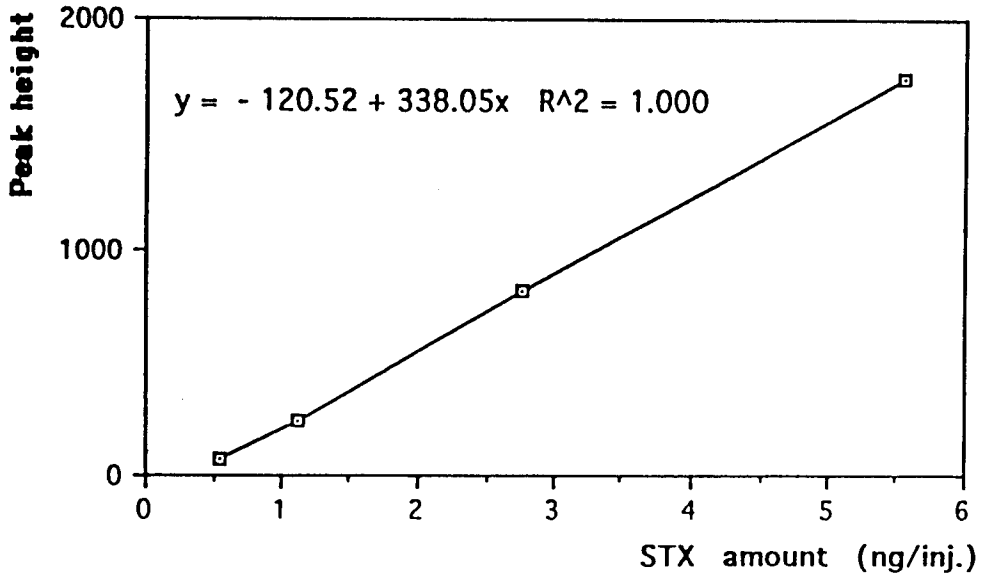


Figure 2. Standard curve used for STX detection by HPLC in rat urine between 0.5 and 5.5 ng per injection. Each value of peak height is an average based on four separate experiments.

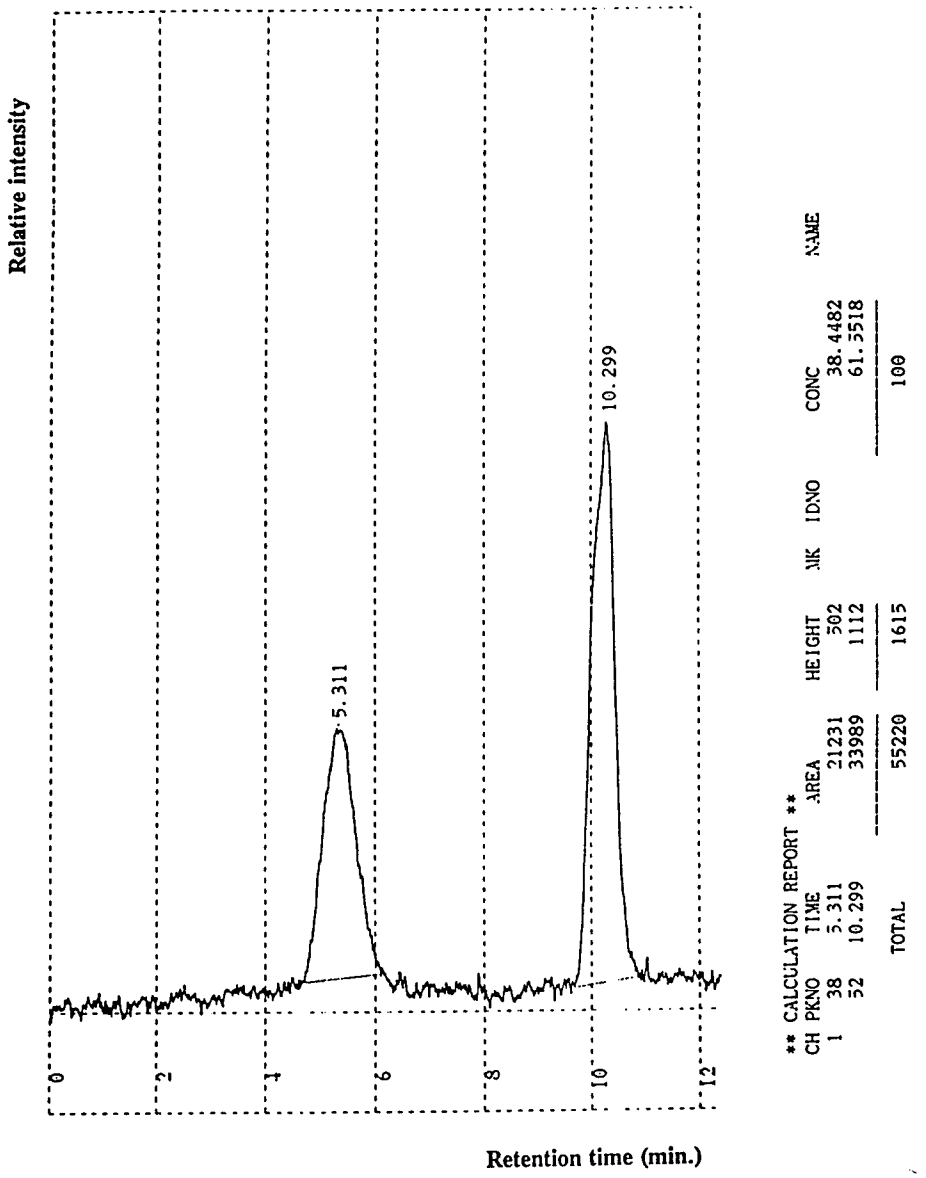


Figure 3. Detection of 23.12 ng/ml (ppb) saxitoxin (retention time 10.29 min) in human urine after resolution and detection by HPLC (fluorescence at wavelength Em=410; Ex=350, attenuation 1).

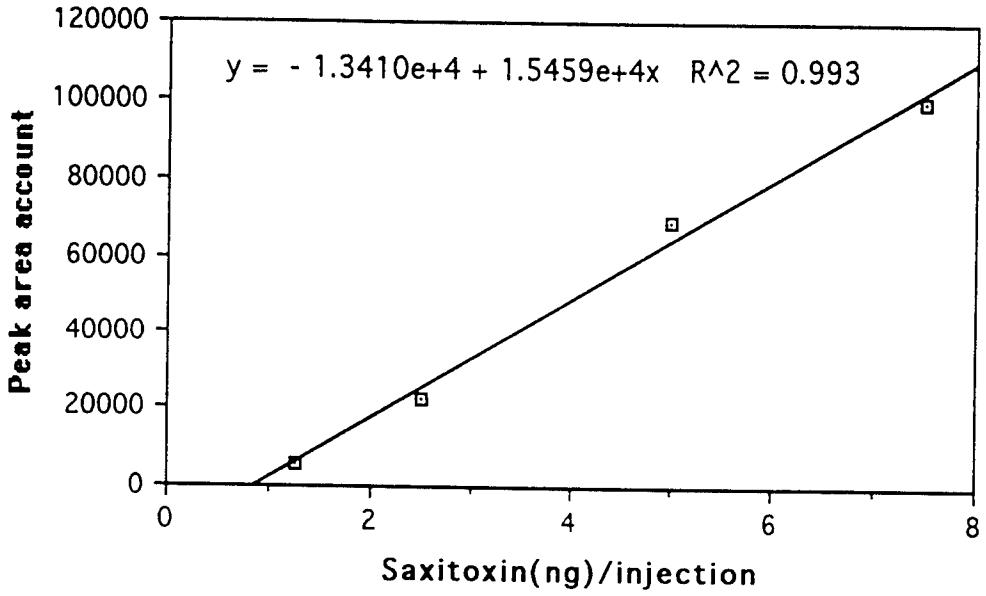


Figure 4. Standard curve of STX in a human urine matrix used for the quantitation of STX in human urine by CF/FAB/MS and a microcapillary HPLC column.

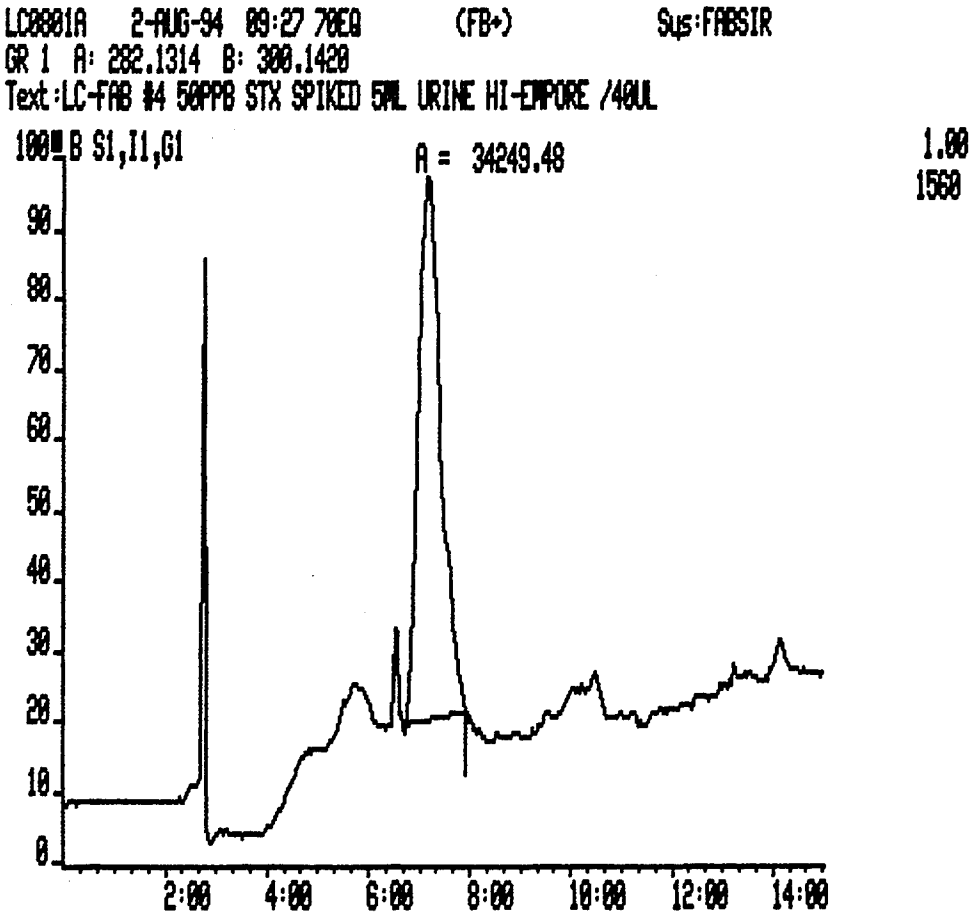


Figure 5. Mass chromatogram of the protonated molecular ion found at m/z+ 300 of STX in a human urine sample to which the equivalent of 50 ppb STX was added. The area count obtained was 34,249 which was equivalent to 3.12 ng STX injected.