ELSEVIER

Journal of Chromatography B, 657 (1994) 119–124

JOURNAL OF
CHROMATOGRAPHY B:
BIOMEDICAL APPLICATIONS

# Method for the identification of saxitoxin in rat urine

Robert G. Stafford*, Harry B. Hines

*Toxinology Division, United States Army Medical Research Institute of Infectious Diseases, Ft. Detrick, Frederick, MD 21702-5011, USA*

(First received October 6th, 1993; revised manuscript received March 15th, 1994)

**Abstract**

Saxitoxin (STX) is one of several related toxins that cause paralytic shellfish poisoning. We used solid-phase extraction (SPE) and prechromatographic oxidation/HPLC with fluorescence detection to isolate, identify, and quantify STX in rat urine. STX recovery from urine with the SPE procedure was approximately $76 \pm 6.5\%$. The standard curve was linear between 2 and 50 ng/ml. The lower limit of quantification with the method was 2 ng STX/ml of rat urine. Preliminary results with i.v. administration of STX to rats demonstrated that this method can detect and quantify STX in urine.

## 1. Introduction

Saxitoxin (STX) is one of several related neurotoxins that cause a very severe, sometimes fatal, form of food poisoning known as paralytic shellfish poisoning (PSP). PSP produces gastrointestinal distress, paresthesias, ataxia, giddiness, muscular weakness, paralysis, and other symptoms. In extreme cases, death may result from respiratory arrest [1–3]. PSP in humans occurs after eating shellfish that fed on toxic dinoflagellates [1,2,4]. Toxins are produced by several dinoflagellate species including *Alexandrium catenella*, *A. tamarensis*, and *Pyridinium bahamense* var. *compressa* [5]. Structures for the major PSP toxins are shown in Fig. 1. Decarbamoylated analogs (those missing the carbamoyl group, $R_4NHCO$) are not shown.

PSP toxins undergo biotransformation in clam and scallop homogenates *in vitro* [6,7]. However, there have been few studies of PSP toxin metabolism in mammals. Hines and coworkers [8] studied STX metabolism and elimination in the rat by injecting tritiated, reduced STX (saxitoxinol, [$^3$H]-STXOL) intravenously. [$^3$H]-STXOL was rapidly excreted unaltered in urine, as determined by HPLC. Approximately sixty percent of the injected STXOL was excreted in the urine within 4 h postinjection. No radioactivity was detectable in feces.

The purpose of this study was to construct a method for analyzing unlabeled STX in urine.

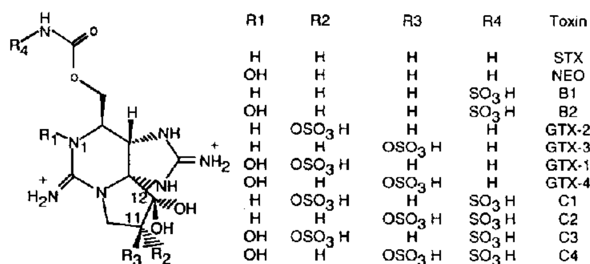


Fig. 1. Structures of PSP toxins.

* Corresponding author.

0378-4347/94/$07.00 © 1994 Elsevier Science B.V. All rights reserved
*SSDI* 0378-4347(94)00180-D

Several analytical methods were developed to monitor PSP toxins in shellfish. These methods included bioassays, immunoassays, and chemical methods (for review of early work see ref. 9). Mirocha *et al.* [10] developed a method for detecting STX in urine based upon continuous-flow fast-atom bombardment mass spectrometry, but the detection limit was too high for *in vivo* analysis. Sullivan *et al.* [11] used a polymeric reversed-phase column to separate PSP toxins and post-column oxidation with periodic acid for fluorescence detection. The STX detection limit was reportedly 4.2 ng/ml (8.4 ng/g shellfish), the mouse bioassay was reported to have a detection limit of 150 ng/ml (299 ng/g shellfish). The post-column oxidation/HPLC procedure separated most of the PSP toxins in shellfish extracts. Previously, we applied one of these monitoring methods, the Sullivan and Wekell HPLC, post-column oxidation method, to rat urine containing STX [12]. Endogenous, unidentified urine interferences were not adequately resolved from STX, rendering the method unsuitable for STX detection in rat urine. The current method that we applied to urine analysis relied upon a solid-phase extraction procedure developed in this laboratory and the prechromatographic, HPLC procedure of Lawrence *et al.* [13] developed for shellfish monitoring. The method will be used to study STX elimination pathways and kinetics *in vivo*. In this study, the rat was used as a model for PSP intoxication. STX was used because it is representative of the PSP toxins, as well as one of the most toxic PSP toxins [4].

## 2. Experimental

### *2.1. Instrumentation*

The HPLC system consisted of two Model 510 pumps, a temperature control module (TCM), a system interface module (SIM), a Model 840 data system (Waters Associates, Milford, MA, USA), an ISS-100 autosampler (Perkin Elmer, Norwalk, CT, USA), LS-4 fluorescence spectrometer (Perkin Elmer), and $V^4$ variable wavelength detector (Isco, Lincoln, NB, USA). Samples were lyophilized with a Model 4.5 freeze dryer (Labconco, Kansas City, MO, USA).

### *2.2. Chemicals and reagents*

HPLC quality water used was obtained from a Milli-Q water system (Millipore, Bedford, MA, USA). All chemicals were analytical or HPLC grade.

### *2.3. Sample preparation*

STX was obtained from Dr. Sherwood Hall, Food and Drug Administration (Washington, D.C., USA) (purity determined to be >96% by HPLC), diluted to 2 mg/ml STX in 0.02 *M* acetic acid and stored at −20°C. STX working dilutions were prepared daily by diluting aliquots of stock solutions with 0.02 *M* acetic acid to the appropriate concentrations in 0.5-ml polypropylene microcentrifuge vials. STX controls were prepared for the SPE procedure by adding variable quantities of STX (working dilutions) to 10 ml of 0.01 *M* sodium acetate (pH 6.9) and adjusting the pH to 8.0 with 6 *M* NaOH. STX standards for recovery data were prepared by adding STX to 3 ml of 1 *M* acetic acid.

Rat urine was collected, filtered, and frozen at −20°C. Urine was used within one month from the collection date. Aliquots (1 ml) were acidified with 167 μl of 1 *M* acetic acid (pH approximately 4.5) to preserve STX. STX was added to acidified rat urine in amounts ranging from 2 to 100 ng/ml immediately before applying to the SPE columns. Urine blanks were prepared as above with the exception that no STX was added.

### *2.4. Solid-phase extraction*

The isolation procedure used in this study utilized an ion-exchange, solid-phase extraction (SPE) of STX. A strong anion-exchange (SAX) column containing a trimethylaminopropyl-bonded phase sorbent was used to remove endogenous urine interferences. STX was isolated from rat urine with a weak cation-exchange

column (CBA) containing a propylcarboxylic acid bonded phase. SAX (0.5 g) and CBA (0.5 g) columns (Bond Elut, Varian, Walnut Creek, CA, USA) were conditioned with 4 ml of methanol, followed by 4 ml of 0.1 *M* sodium acetate (pH 7.5). SAX and CBA columns were attached in series and a 20-ml reservoir was attached to the top of the SAX column. Assembled columns were placed on the SPE vacuum manifold (Supelco, Bellefonte, PA, USA).

STX-amended urine samples or STX controls, prepared as described above, were placed in the 20-ml reservoirs. Vacuum was applied to the column manifold (7–34 kPa). Flow was maintained at 1 drop per second or less and controlled by adjusting sample needle valves. The samples passed first through the SAX columns directly into the CBA columns. Two ml (0.75 column volumes) of 0.1 *M* sodium acetate (pH 7.5) were applied after the samples reached the top of the SAX column beds to elute any remaining STX from the SAX columns onto the CBA columns. SAX columns were then discarded. Next, CBA columns were rinsed with 6 ml deionized water (3 column volumes). STX was eluted from the CBA columns with 3 ml (1 column volume) of 1 *M* acetic acid as described above. Samples were collected in 17 × 100 mm polypropylene test tubes and lyophilized overnight. STX standards prepared for recovery experiments were not subjected to the SPE procedure but were lyophilized for 16 h.

### 2.5. Oxidation procedure

The oxidation procedure of Lawrence and Ménard [14] was modified slightly for urine analysis. Oxidation reagent was prepared fresh daily by adding 50 μl of 10% $H_2O_2$ to 500 μl of 1 *M* NaOH. Lyophilized samples were reconstituted with 100 μl of deionized water and mixed at high speed for 3–4 s (Vortex Jr. Scientific Products, McGaw Park, Il, USA). Freshly prepared oxidation reagent (68 μl) was added to samples (100 μl), which were then mixed for 3–5 s. After a 2-min incubation period, the reaction was stopped with 5 μl of glacial acetic acid. Oxidized samples were filtered with 0.22-μm microcentrifuge filters (PGC Scientific, Gaithersburg, MD, USA) at approximately 6000 *g* for 15 min in an IEC Centra 4B centrifuge (International Equipment Co., Needham Heights, MA, USA).

### 2.6. HPLC conditions

Oxidized STX was injected onto a reversed-phase HPLC system coupled to a fluorescence detector, using the conditions described by Lawrence and Ménard [14]. In addition, UV absorbance of the oxidized STX was monitored at 335 nm [15]. Volumes of 100 μl were injected onto the HPLC. HPLC run times were 60 min. The column used was a 15 cm × 4.6 mm I.D. Supelco LC-18 column ($C_{18}$, 3 μm particle size). Column temperature was maintained at 30°C. Mobile phases consisted of (A) 0.1 *M* ammonium formate, pH 6.0, and (B) acetonitrile. The column was equilibrated with 0.1 *M* ammonium formate for at least 20 min before each injection. The gradient consisted of linear segments: 100% A to 95% A in 15 min; hold for 10 min; from 95% A to 0% A in 13 min for column cleaning; and back to 100% A in 7 min for column equilibration.

The fluorescence spectrometer was set to an excitation wavelength of 346 nm, 5 nm slit width, and an emission wavelength of 408 nm, 5 nm slit width. Optimal wavelengths were determined by separate emission and excitation scans over a short wavelength range before the start of the experiment. Sigmaplot (version 4.04, Jandel Scientific, Corte Madera, CA, USA) was used to plot standard curves and perform least squares regression analysis on calibration data.

### 2.7. In vivo experiment

Three male Fischer VAF/Plus rats weighing approximately 200–250 g each were injected with 2 μg STX/kg (1 μg STX/ml) in the dorsal penile vein. One control rat was injected with sterile saline. The rats were held in plastic metabolism cages (Nalge Co., Rochester, NY, USA) for 144 h post injection. Urine was collected at 4-h

intervals for the first 24 h, at 30 h, then at 24-h intervals thereafter. Prior to placing rats in their cages, 1 *M* acetic acid was added to the urine collection cups (250 $\mu$l per 4-h collection) to acidify the urine and preserve STX. Urine samples were frozen, processed using the SPE method described, and analyzed using the pre-chromatographic oxidation, HPLC method.

## 3. Results and discussion

STX recovery from rat urine with the SPE procedure was determined. Recovery was calculated by dividing the average area of the oxidized STX peak from urine by the average area of the oxidized STX peak in the STX standard and multiplying by 100. The results are shown in Table 1. Analysis of the column washes and eluates demonstrated that STX was not present. It appeared that the remaining STX was still bound to the CBA column. An additional column volume (3 ml) of 1 *M* acetic acid failed to elute any remaining STX from the CBA columns. Mirocha and coworkers [10] used CBA ion-exchange columns to extract 20 $\mu$g of STX from 2 ml human urine. They reported preliminary recovery data of 75%, which is comparable to our recoveries with rat urine.

STX isolated from rat urine was identified by comparing retention times with STX standards.

Table 1
STX recovery with the double ion-exchange SPE procedure

| STX concentration | 1 ml STX amended rat urine [a] | STX controls [b] |
|---|---|---|
| 2 ng/ml | 63.3% (4) [c] | 56.4% (5) |
| 10 ng/ml | 81.9% (5) | 73.2% (5) |
| 25 ng/ml | 83.4% (5) | 75.8% (6) |
| Avg. recovery ± S.E. | 76.2 ± 6.5% | 68.5 ± 6.1% |

[a] Average recovery: percent ± standard error.
[b] Not significantly different from STX-amended rat urine ($\alpha$ = 0.05).
[c] Numbers in parentheses denote replicates.

Data from non-amended rat urine samples was used to identify urine interferences. A typical chromatogram for a 25 ng/ml STX standard that was lyophilized and oxidized, but not subjected to the SPE procedure, is shown in Fig. 2A. Representative chromatograms for STX-amended (2 ng/ml STX) and non-amended rat urine samples (0 ng/ml STX) subjected to the

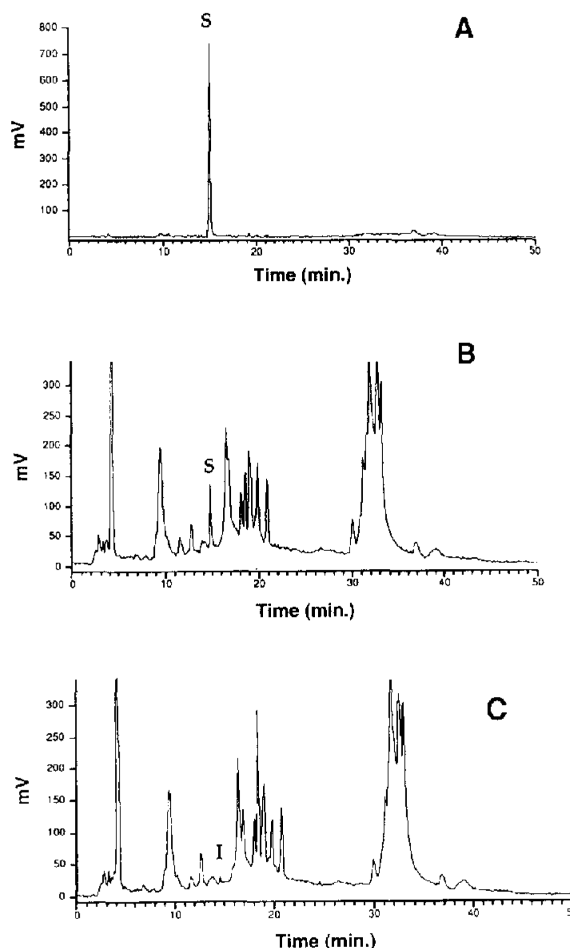


Fig. 2. Typical chromatograms depicting (A) 100-$\mu$l injection of 25 ng/ml oxidized STX standard, (B) 1 ml of acidified rat urine amended with 2 ng STX and processed with the SPE procedure (100-$\mu$l injection), and (C) acidified rat urine (1 ml) without STX (urine blank) processed with the SPE procedure, (100-$\mu$l injection). Oxidized STX peak is marked with an S, and a small interfering peak present in the urine blank is marked with an I. Fluorescence detection was used. Wavelengths were 346 nm and 408 nm for excitation and emission, respectively.

SPE and prechromatographic oxidation HPLC procedure are shown in Fig. 2B and C, respectively. The average signal-to-noise ratio (S/N) was estimated to be approximately 8.0 for 2 ng/ml of STX-amended rat urine samples. The reversed-phase HPLC procedure adequately separated most interferences from oxidized STX in rat urine. We found that oxidized STX was not retained on the HPLC column from injection to injection (memory effect) at levels up to 100 ng/ml. Above 100 ng/ml, memory effects were increasingly evident.

A standard curve for STX-amended urine samples was constructed by plotting oxidized STX peak areas against the amount of STX added to 1 ml of rat urine. The curve was linear from 2 to 50 ng/ml ($y = 2.13 \cdot 10^5 x + 8.28 \cdot 10^4$, $r = 0.9966$). While 2 ng STX/ml rat urine was determined to be the lower level of quantification, STX was detectable in the mid pg/ml range. Data for 100 ng/ml STX indicated that the detector saturated at the settings used. Diluting samples, using smaller injection volumes, or reducing detector sensitivity would allow us to detect larger amounts of STX in rat urine.

Statistical tests were used to compare the STX controls (subjected to SPE) and STX-amended rat urine samples. The sample variances were tested using an F-test [16] to determine if, for each corresponding concentration (2, 10, 25 ng/ml), sample variances were statistically the same or different from one another. We found that the sample variances were not statistically different ($\alpha = 0.05$) for the 2 and 10 ng/ml concentrations. However, for the 25 ng/ml STX concentrations, sample variances of the STX controls and the STX-amended rat urine samples were significantly different ($\alpha = 0.05$). Based on these results, means of the STX controls and the STX-amended rat urine for the 2 and 10 ng/ml concentrations were tested using a t-test (independent samples, equal variances) [16]. The means of the STX controls and the STX-amended rat urine for 25 ng/ml concentrations were tested using a modified t-test [16]. We found that in all cases the means of each set of data at a particular STX amount were not statistically different ($\alpha = 0.05$). Therefore, we concluded that STX recovery from rat urine was not different from STX recovery from an aqueous solution (0.01 *M* sodium acetate) with this SPE procedure.

Table 2
STX identification in rat urine after intravenous injection

| Time post injection (h) | Average [STX] [a] | Average total STX excreted [b] | Average percent of dose excreted |
|---|---|---|---|
| 4 | 51.9 ± 14.2 | 57.2 ± 8.3 | 13.3 ± 2.2 |
| 8 | 43.3 ± 13.2 | 51.2 ± 18.4 | 11.9 ± 4.3 |
| 12 | 37.2 ± 8.9 | 50.7 ± 24.1 | 12.0 ± 5.9 |
| 16 | 9.0 ± 2.6 | 25.6 ± 13.1 | 6.0 ± 3.2 |
| 20 | 10.1 ± 1.2 | 20.9 ± 6.1 | 4.9 ± 1.5 |
| 24 | 14.3 ± 7.6 | 21.4 ± 6.9 | 5.0 ± 1.6 |
| 30 | 8.1 ± 2.6 | 17.8 ± 5.0 | 4.1 ± 1.1 |

[a] ng/ml ± S.E., $n = 3$.
[b] ng ± S.E., $n = 3$.

Preliminary results were obtained for rats injected intravenously via the dorsal penile vein. Three rats received STX doses of 2 $\mu$g/kg each. Results are shown in Table 2. STX was detectable in rat urine up to 144 h post injection, but quantifiable up to 30 h. Fig. 3 is a representative chromatogram for urine collected from one rat at 20 h post injection. The STX peak was evident at

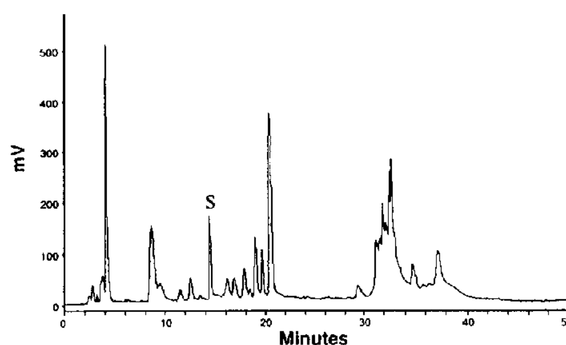


Fig. 3. Chromatogram from *in vivo* STX experiment. Rat (208 g) received 2 $\mu$g STX/kg i.v. This chromatogram is from the 20 h time period. Acetic acid (0.25 ml, 1 *M*) was added to the urine collection cup. Corrected urine volume for the time period was 2.63 ml. A 1-ml urine aliquot was processed as described. Concentration of STX found in the urine was 13.58 ng/ml (35.7 ng total) which corresponds to 8.5% of the total dose (416 ng) given. Retention time for the STX peak was 14.44 min.

a retention time of 14.44 min and was identified by comparing its retention to that from a standard. These preliminary results demonstrated that STX was detectable and quantifiable with the developed method. The results also showed that STX was excreted rapidly in the urine after intravenous injection, as was found for [$^{3}$H]-STXOL injected intravenously [8].

Using the prechromatographic oxidation/HPLC procedure, Lawrence and Ménard [14] reported a detection limit of 3–6 ng STX/g of shellfish (1.5–3 ng/ml, 20 $\mu$l injection). They used the procedure outlined in the AOAC, *Official Methods of Analysis* [17] to prepare shellfish extracts. They used SPE $C_{18}$ cartridges to clean up the extracts and ion-exchange extraction to separate several of the toxins. The method also required two different oxidation procedures for optimal sensitivity of all PSP toxins. Lawrence and Ménard also evaluated two additional methods of analysis for PSP toxins using samples of shellfish and found these methods compared reasonably well with the prechromatographic, HPLC method. The post-column method of Sullivan *et al.* [11] tended to report higher values of PSP toxins than the prechromatographic method. The mouse bioassay tended to underestimate PSP concentrations. These authors postulated that the salt effect [18] influenced the mouse bioassay. They also hypothesized that post-column oxidation results were higher because hydrolysis of B and C toxins to STX and neosaxitoxin (NEO) occurred during storage prior to LC analysis. Lawrence and Ménard reported an approximately 5 times higher sensitivity for STX than the Sullivan/Wekell post-column method. We found the detection limit for our method ($<2$ ng/ml rat urine) compared favorably with the method of Lawrence and Ménard.

It is not known at this time if this method can detect the presence of possible STX metabolites or other PSP toxins. Although the procedure outlined in Lawrence *et al.* [13] and Lawrence and Ménard [14] was adequate for detecting many PSP toxins in shellfish, the potential for detecting other PSP toxins in rat urine is yet unproven. It is likely that our SPE procedure would require modification to efficiently extract other PSP toxins or metabolites from rat urine.

The method described in this study is applicable to the demanding task of measuring sublethal quantities of STX in rat urine and will permit us to study STX elimination in the rat. Preliminary results from the administration of sublethal doses of STX to rats indicated the applicability of this method.

## References

[1] K.F. Meyer, *N. Engl. J. Med.*, 249 (1953) 843.
[2] M.D. Ahles, *Am. J. Public Health*, 64 (1974) 807.
[3] D.C. Rodrigue, R.A. Etzel, S. Hall, E. De Porras, O.H. Velasques, R.V. Tauxe, E.M. Kilbourne and P.A. Blake, *Am. J. Trop. Med. Hyg.*, 42 (1990) 267.
[4] Y. Shimizu, in A.T. Tu (Editor), *Handbook of Natural Toxins*, Vol. 3. Marcel Dekker, New York, 1988, Ch. 3, p. 63.
[5] D. Mebs and F. Hucho, in W.T. Shier and D. Mebs (Editors), *Handbook of Toxinology*, Marcel Dekker, New York, 1990, Ch. 7, p. 493.
[6] Y. Shimizu and M. Yoshioka, *Science*, 212 (1981) 547.
[7] J.J. Sullivan, W.T. Iwaoka and J. Liston, *Biochem. Biophys. Res. Comm.*, 114 (1983) 465.
[8] H.B. Hines, S.M. Naseem and R.W. Wannemacher, Jr., *Toxicon*, 31 (1993) 905.
[9] J.J. Sullivan, M.M. Wekell and S. Hall, in A.T. Tu (Editor), *Handbook of Natural Toxins*, Vol. 3, Marcel Dekker, New York, 1988, Ch. 4, p. 87.
[10] C.J. Mirocha, W. Cheong, U. Mirza and Y.B. Kim, *Rapid Commun. Mass Spectrom.*, 6 (1992) 128.
[11] J.J. Sullivan, M.M. Wekell and L.L. Kentala, *J. Food Sci.*, 50 (1985) 26.
[12] H.B. Hines, personal communication, 1993.
[13] J.F. Lawrence, C. Ménard, C.F. Charbonneau and S. Hall, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 404.
[14] J.F. Lawrence and C. Ménard, *J. Assoc. Off. Anal. Chem.*, 74 (1991) 1006.
[15] H.A. Bates, R. Kostriken and H. Rapoport, *J. Agric. Food Chem.*, 26 (1978) 252.
[16] R.G.D. Steel and J.H. Torrie, *Principles and Procedures of Statistics: A Biometrical Approach*, 2nd edn., McGraw-Hill, New York, 1980, Ch. 5, p. 86.
[17] *Official Methods of Analysis of the Association of Official Analytical Chemists*, Associaton of Official Analytical Chemists, Arlington, 1984, sec. 18.086-18.092.
[18] E.J. Shantz, J.D. Mould, D.W. Stanger, J. Shavel, F.J. Riel, J.P. Bowden, J.M. Lynch, R.S. Wyler, B. Riegel and H. Sommer, *J. Am. Chem. Soc.*, 79 (1958) 5230.