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## Note

# Thin-layer chromatography with flame ionization detection for the determination of tetrodotoxin in biological fluids*

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Tetrodotoxin (TTX) is one of the best known marine toxins because of its frequent involvement in fatal food poisoning, its unique chemical structure and its specific action of blocking sodium channels of excitable membranes. The etiology of TTX is now one of the most interesting topics in the field of public health because of its wide distribution among genetically unrelated animals and the marked variability of toxicity in one species [1–4]. Therefore, a simple and reliable method for the determination of TTX in biological materials is desirable.

Recently, flame ionization detection (FID) has been widely applied to types of chromatography other than gas chromatography [5]. Cotgreave and Lynes [6] fed a vaporized sample from a narrow chromatoplate to the detector. Further approaches in this area were conceived by Padley [7], Szakasits et al. [8] and Mukherjee et al. [9]. Padley's idea of using a rod as a support instead of a plate was developed further by Okumura and Kadono [10]. The combined use of thin-layer chromatography with flame ionization detection (TLC–FID) now represents a significant advance as a method applicable to screening in the pharmaceutical and biomedical fields [11–14]. The sensitive, relatively simple and rapid procedure also offers advantages in analyses of heavy oil and food additives and in industrial research analyses. However, there has been no attempt to determine TTX in biological materials using TLC–FID.

In this paper we describe a method for the determination of TTX in serum and urine by TLC–FID for the study of TTX poisoning in public health and forensic medicine. We also discuss the accuracy and reliability of the TLC–FID system compared with conventional methods.

*This paper is dedicated to Emeritus Prof. Dr. T. Kita.

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## EXPERIMENTAL

### *Materials*

Tetrodotoxin Crystalline 3X was purchased from Sankyo (Tokyo, Japan). O,O-Dimethyl S-(N-methylcarbamoylmethyl)phosphorodithioate was obtained as an internal standard (I.S.) from Wako (Osaka, Japan). Organic solvents were of special reagent grade. Other reagents were of analytical-reagent grade or of the highest quality commercially available, and were used without further purification.

The serum samples used for spiking were obtained from post-mortem cases in which TTX was not implicated and from the Department of Clinical Laboratory, Tottori University School of Medicine. The urine samples were collected from volunteers. These samples were stored at $-70°C$ until analysis.

### *Analytical methods*

Purification of TTX was carried out as described previously [15] by successive treatments of extracts on a column of Amberlite IRC-50. A 5-ml aliquot of a serum or urine sample was extracted twice with 10 ml of 0.5% acetic acid. After filtration, the filtrate was concentrated and ethanol was added. The precipitate was then removed and the filtrate was again concentrated. After adding three volumes of Folch's solvent [chloroform–methanol (2:1)], the mixture was stirred and centrifuged at 2000 *g* for 10 min. The upper layer was concentrated under reduced pressure to remove methanol and the residue (aqueous layer) was passed through a column (10 cm × 1.8 cm I.D.) packed with Amberlite IRC-50 ($NH_4^+$). The column was washed with distilled water and the toxin was eluted with 10% acetic acid. The eluate was evaporated to dryness in a dry block (M&S Instruments, Osaka, Japan). The residue was dissolved in 50 $\mu$l of 1% acetic acid and 50 $\mu$l of I.S. solution. After centrifugation at 1500 *g* for 5 min, a 5-$\mu$l portion of the supernatant was used for TLC–FID. The extract prepared from serum was freed from lipids by extraction with diethyl ether prior to passage through the Amberlite column.

### *Apparatus and operating conditions*

TLC–FID was carried out with an Iatroscan TH-10 (Iatron Labs., Tokyo, Japan), which was connected to a Chromatopac-E1A integrator system (Shimadzu, Kyoto, Japan) and an 056 recorder (Hitachi, Tokyo, Japan). Glass developing tanks (Iatron) were lines with Whatman 1CHR filter-paper which was pre-washed with mobile phase. The stationary phase was Chromarod S II (Iatron), which was a quartz rod coated with silica gel, and the mobile phase was *n*-butanol–acetic acid–water (60:15:30, v/v). The scanning speed was 30 s/scan, the flow-rate of hydrogen was 160 ml/min and that of air was 2000 ml/min. The Chromarods were stored in groups of ten in the rod holder provided with the instrument. For activation, all rods were passed through the flame ionization detector three times prior to use. A 5-$\mu$l aliquot of sample was then applied at the origin of the rod. After the run, the rods were dried for 30 min in a rod dryer (Iatron) at 140°C and transferred to the scanning frame for detection.

## RESULTS AND DISCUSSION

### *Separation and internal standard*

Many developing solvents have been applied to the detection of TTX by TLC [16–22]. The mobile phase *n*-butanol–acetic acid–water (60:15:30) appeared to be the most suitable for the determination of TTX by TLC–FID. For the purpose of standardization, a number of compounds that were chemically similar to TTX were tested. Of those tested, O,O-dimethyl S-(N-methylcarbamoylmethyl)-phosphorodithioate proved to be the most suitable as an I.S. An I.S. solution was prepared by dissolving 160 mg of O,O-dimethyl S-(N-methylcarbamoylmethyl)-phosphorodithioate in 50 ml of distilled water. The compounds showed good peak shapes and were well resolved in the central zone of the chromatogram, all of which provided a realistic basis for good reproducibility of analysis. No significant interference from other substances present in the sample solutions was observed. The TLC–FID separation of TTX in the presence of the I.S. is shown in Fig. 1.

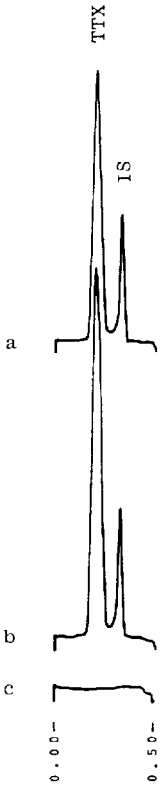


Fig. 1. TLC–FID separation of TTX in serum and urine. (a) Serum containing 1.5 μg of TTX and 8.0 μg of I.S.; (b) urine containing 2.0 μg of TTX and 8.0 μg of I.S.; (c) blank. The values 0.00 and 0.50 are retention times (min).

*Calibration graph*

The calibration graph for the determination of TTX was obtained by plotting the peak-area ratio of TTX to I.S. against the known amount of added TTX. There was a linear relationship between the peak-area ratio and the amount of TTX in the range 0.2–2.0 μg per sample used. The equation parameters were $y=1.060x-0.038$ ($r=0.998$, $n=16$). Under the above conditions the detection limit was 0.2 μg in 5 ml of serum or urine.

*Accuracy and reproducibility*

In order to calculate the recovery, TTX was added to TTX-free serum and urine from non-poisoning cases. The reproducibility of TLC–FID was examined by replicate analyses of ten samples of the serum and urine. The mean value, standard deviation and recovery are summarized in Table I. It is also demonstrated in Tables II and III that the relative retention time (RRT) is more useful

TABLE I

REPRODUCIBILITY OF THE DETERMINATION OF TTX IN SERUM AND URINE

| Sample | Spiking level (μg) | Relative recovery* (%) | | |
|---|---|---|---|---|
| | | Mean | S.D. | C.V. |
| Serum | 2.0 | 77.4 | 3.14 | 4.06 |
| Urine | 2.0 | 85.9 | 3.10 | 3.61 |

*Ten determinations at each level.

TABLE II

WITHIN-DAY PRECISION OF THE DETERMINATION OF TTX IN SERUM AND URINE

| Rod* | Distance travelled (mm) by | | $R_F$ |
|---|---|---|---|
| | TTX | Solvent front | |
| 1 | 40.5 | 104.3 | 0.388 |
| 2 | 41.1 | 101.5 | 0.405 |
| 3 | 38.8 | 102.7 | 0.378 |
| 4 | 43.3 | 107.2 | 0.404 |
| 5 | 42.9 | 109.8 | 0.391 |
| 6 | 39.9 | 98.4 | 0.405 |
| 7 | 40.6 | 102.1 | 0.398 |
| 8 | 43.4 | 110.6 | 0.394 |
| 9 | 41.3 | 97.7 | 0.422 |
| 10 | 39.8 | 103.1 | 0.386 |
| Mean | | | 0.397 |
| S.D. | | | 0.013 |
| Coefficient of variation (%) | | | 3.274 |

*Nos. 1–5, serum samples; Nos. 6–10, urine samples.

TABLE III

WITHIN-DAY PRECISION OF THE DETERMINATION OF TTX IN SERUM AND URINE

| Rod* | Retention time (min) | | RRT** |
|---|---|---|---|
| | TTX | I.S. | |
| 1 | 0.203 | 0.333 | 0.610 |
| 2 | 0.206 | 0.334 | 0.617 |
| 3 | 0.194 | 0.302 | 0.642 |
| 4 | 0.217 | 0.361 | 0.601 |
| 5 | 0.215 | 0.348 | 0.618 |
| 6 | 0.199 | 0.322 | 0.618 |
| 7 | 0.203 | 0.333 | 0.610 |
| 8 | 0.217 | 0.347 | 0.625 |
| 9 | 0.206 | 0.342 | 0.602 |
| 10 | 0.199 | 0.322 | 0.618 |
| Mean | | | 0.616 |
| S.D. | | | 0.012 |
| Coefficient of variation (%) | | | 1.948 |

*Nos. 1–5, serum samples; Nos. 6–10, urine samples.
**Relative retention time.

than $R_F$. These results show that the method is applicable for forensic analytical purposes.

*Evaluation of the analyses*

To our knowledge the method described here is the first report of a TLC–FID system for the determination of TTX, rather than 2-amino-6-hydroxymethyl-8-hydroxyquinazoline ($C_9$-base), which is derived from TTX by alkaline degradation.

Some chemical assay techniques have previously been developed. The mouse assay method [23] for monitoring purposes is rapid and simple, but the development of instrumental analysis methods was desirable in order to avoid the use of a large number of mice and to improve the accuracy, sensitivity and specificity. Chromatographic methods have also been used. TLC [16–22] has been adapted for detection of underivatized TTX, but quantification is less straightforward. Gas chromatography, gas chromatography–mass spectrometry [15,24], and high-performance liquid chromatography (HPLC) [25,26] are much more sensitive. These methods need difficult chemical modifications, which involve complicated steps to obtain $C_9$-base from TTX by alkaline degradation before analysis. A further problem with HPLC is that it is not adapted to include an I.S. for quantification.

The TLC–FID method described here requires no derivatization. This method provides superior sensitivity and accuracy, and does not require the animals used in the mouse bioassay. The simple and convenient determination of TTX in a variety of marine organisms is becoming increasingly important from the standpoint of public health and forensic toxicology, as food poisoning from ingestion

of the toxin is often fatal to man. The technique proposed here is efficient for identifying and determining this compound in biological materials, even though some complexity [15] still exists in the purification of the toxin, which is necessary to remove the diverse interfering substances.

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