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Effect of Different Treatments on Excretion of Tetrodotoxin after Oral Administration in Rat

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Abstract

The levels of tetrodotoxin (TTX) in the serum and urine were investigated by using LC-MS method in healthy male wistar rats after oral administration with single dose of 60 nmoleTTX mixture. After treatment, male wistar rats were randomly divided into three groups: (1) rats drink distilled water; (2) rats drink saline solution; (3) rats drink alkaline electrolyzed (AE) water. The serum and urine samples were collected at intervals between 2 hr to 60 hr after orally administration. It was found that TTX can be detected from serum and urine up to 60 hr the max that was tested when intoxicated poisoning occurred. The highest amount of TTX appeared after 4 hr and 6 hr in the serum and urine, respectively. When the rats took AE water, TTX was secreted earlier from the serum and urine. It indicates that AE water enhances the excretion of TTX in the rats. Moreover, LC-MS is useful for detecting TTX in serum and urine samples and may have diagnostic potential for TTX intoxication.

Keywords: Tetrodotoxin (TTX); Excretion; Rat; LC-MS

Introduction

Outbreaks of tetrodoxin (TTX) food poisoning are still reported in various countries including Taiwan [1-6]. Until now the causative agent of these food poisonings was identified from the toxin in food. However, the suspected food was sometimes discarded and no leftover was retained. Hence, TTX diagnosis from patients is very important. TTX is a heterocyclic guanine compound, and acts by blocking the flux of sodium ions through voltage-gated sodium channels on excitable cell membranes [5].

In the past, the principle impediment to analyze TTX in the serum and urine was lack of an uncomplicated and reproducible method with sufficient sensitivity to detect the picogram to nanogram quantities thought to be present [7,8]. Hence, a new approach for examining TTX in the serum and blood was established by using C18 cartridge column, ultrafiltration and liquid chromatography- mass spectrometry (LC-MS) [9-15].

The purpose of this study was to establish a method for detecting trace concentration of TTX in the serum and urine of exposed rats in order to determine the time course of excretion and to determine the effectiveness of different supportive treatments. The results are suspected to be useful for enhancing potential method for TTX excretion in the patient.

Materials and Methods

Materials

The dried powder of mixed authentic tetrodotoxin (TTX),4-epiTTX and anhydro-tetrodotoxin (anh-TTX) were obtained from Wako Pure Chemical Industries (Tokyo, Japan). The purity of TTX was 94%. The toxins were dissolved with 0.03 M acetic acid and kept at-20°C until use. Male Wister rats (n=24) weighing between 210 g and 260 g (234 g ± 12 g) were purchased from National Laboratory Animal Centre, Taipei.

Experimental treatment of rats

Male Wister (n=18) rats were fed individually in stainless steel wire bottom cages with a controlled environment (25°C, 50%-60% humidity, 12 hr light per day) for 2 weeks. The animals were fed a laboratory diet (PMI Feeds, USA). Experimental rats were each orally administered with a single dose of 60 nmole TTX (19.14 μg), which is toxic to lower than lethal dose to rats and is previously obtained by using rat bioassay to substitute mouse assay [14]. Then these rats were divided into three groups supplied with distilled water (as control), saline solution and alkaline electrolyzed (AE) water in free access, respectively. The saline solution was dissolved 0.9% NaCl in distilled water. The AE water (pH=9.0) was made by an electrolyzer (ROX-20TA, Hoshizaki Electric Co. Ltd., Toyoake, Aichi, Japan). Immediately after dosing, animals were transferred to metabolic cages, and the blood and urine samples were collected separately at 2hr, 4hr, 6hr, 8hr, 12hr, 24hr, 36hr, 48hr and 60 hr after dosing. The blood of rats were taken from the tail veins of each rat. The serum of blood samples were collected by centrifugation (2,000 x g for 15 min). The serum and urine samples were frozen at -20° until assay was carried

out. The other 6 rats were used to collect the serum and urine for establishing the standard curves and recoveries of TTX in the serum and urine by spiked with authentic TTX.

Sample preparation

The sample cleanup procedure used was based on the procedure developed by Andrinolo et al. [12]. Each (1.0 ml) of the serum and urine samples was thawed and mixed with 200 μl of 0.5 M acetic acid to keep it acidic. The samples were immediately centrifuged at 10,000×g for 10 min. The supernatant was passed through a 3 ml prepared cartridge column (C18 Sep-Pak cartridges, particle size 37μm-55 μm, sorbent weight 500 mg, Millipore, Waters, MA). The prepared cartridges were previously washed with 10 ml methanol and then 10 ml water before use. After applying sample into the cartridge, 10 ml 0.3% acetic acid was eluted. The eluant was freeze-dried, dissolved in 2 ml 0.3% acetic acid and filtered through a 3,000MW cut-off Ultrafree microcentrifuge filter (Micron YM-3, Millipore, Waters). The filtrate was freeze-dried and dissolved in a small amount (1 ml) of distilled water.

LC-MS analysis for TTXs

The chromatographic system was composed of a Waters Model 2690 quaternary LC pump coupled analysis. TTX was performed on a reverse-phased column (Puresil C-18 GP, 4.6 mm× 250 mm, Waters) and the mobile phase was aqueous 30 mM heptafluorobutyric acid-1 mM ammonium acetate (pH 5.0) at flow rate of 1.0 ml/min for TTX. The injection volume was 10 μl. The ZMD 2000 mass spectrometer equipped with an atmospheric pressure ion source and an electrospray ionization (ESI) interface (Finnigan MAT, CA, USA) was employed for detection. The electrospray interface was typically operated using the following setting fragmentor, 150 V; nebulizer, N2 (60 psi); dry gas N_2 (10 l/min, 350°C; V-cap, 3,500 V [9-11].

Standard curves, detection limit and recovery

The standard curve of TTX was prepared from 1 ng/ml, 5 ng/ml, 10 ng/ml, 50 ng/ml, 100 ng/ml of TTX. The determination was performed three times (n=3). Data from the curve were subjected to linear regression analysis and peak area (y) versus amount of TTX (x) was platted. The detection limit that was evaluated according to the rates of sample peak area to noise area was more than three times. Spiked samples were prepared by dissolving approximate 1μg, 5μg, 10 μg of TTX in normal serum and urine (1.0 ml). The sample were mixed with 500 μl of 0.5 M acetic acid, centrifuged, passed through the cartridge column, and extracted with methanol as described above.

Statistical analysis

Statistical analysis for differences among rats in the experimental groups was performed by the one-way analysis of variance (ANOVA). When the ANOVA identified differences among groups, multiple comparisons among means were made using Duncan's new multiple range tests. A $p < 0.05$ was considered statistically significant.

Results

When using LC-MS to determine TTX toxin, the standard curve was linear in the range of 1-100 ng/ml for serum and urine. The linear equations were y=817x+2190 (r=0.996) and y =11.78x+1143.38 (r=0.992) for serum and urine, respectively. The recovery of TTX in

the serum and urine were 91.2% and 94.3%, respectively. The detection limit was 0.5 ng/ml of TTX. Figure 1 contains LC-MS chromatograms in selected ions at m/z 302 and 320 for standard TTX, 4-epiTTX and anh-TTX, respectively.

Spiked samples were prepared by dissolving 18 nmole of TTX in the serum and urine (Figure 2). Figure 3 shows LC-MS chromatograms of the serum and urine samples of rat (4 hr). The rats were orally administered with a dose of 60 nmole of TTX, the serum and urine samples were collected during a 60 hr experimental course. The LC-MS could detect TTX amount in serum up to 36 hr and in the urine up to 60 hr. It was found that the highest amount of TTX appeared after 4 hr and 6 hr in the serum and urine, respectively. Hence, TTX can be detected from serum up to 36 hr and from urine up to 60 hr when intoxicated poisoning occurs. After 2 hr, TTX was secreted from the urine. The maximum peaks of TTX appeared at 6 hr in urine when the rats took each kind of water. By the end of the collection period (60 hr), almost TTX was excreted from the urine. Hence, TTX could be perfectly secreted after 60 hr of food poisoning.

The effect of other water (saline solution and AE water) on TTX metabolism in rats was also evaluated in this study. When the rats took saline solution and AE water, the time course of TTX excretion in rats is shown in Figure 4. When the rats took AE water, it was found that TTX could be secreted earlier from the urine at 4 hr. After 2 hr, TTX was secreted into the urine and clearly secreted after 60 hr. It appears that administration of AE water could stimulate a more rapid excretion of TTX through the urine (Figure 5).
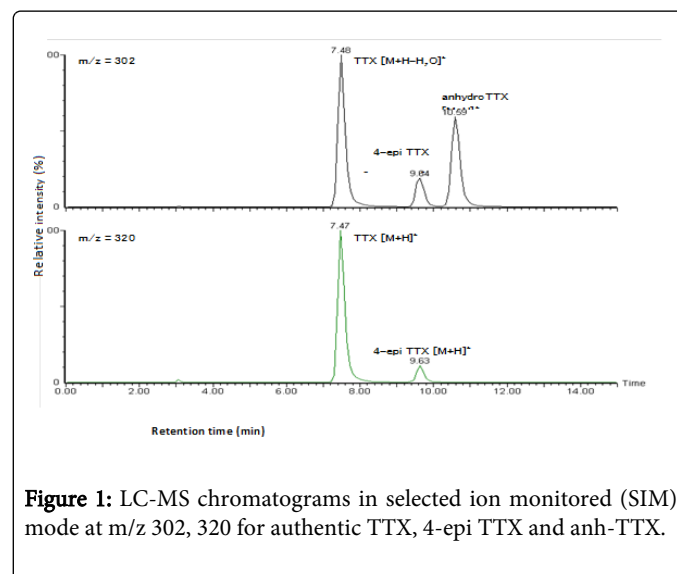

Figure 1: LC-MS chromatograms in selected ion monitored (SIM) mode at m/z 302, 320 for authentic TTX, 4-epi TTX and anh-TTX.

Discussion

This is the first experimental effort to resemble TTX toxin incidents in rats. We have developed a simple technique for the determination of TTX in serum and urine of humans [9-11]. In this study, the unambiguous identification of TTX toxin in the rat was obtained using LC-MS method. The technique was applied to experimentally exposed animals and proved to be useful in confirming the toxins in even the smallest amount was demonstrated. LC-MS is quite rapid technique and has been considered as possible replacement for the mouse bioassay [13-17].
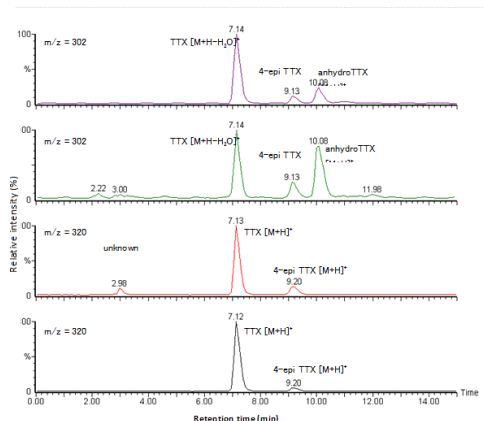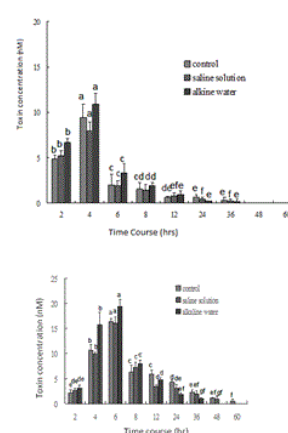
Figure 2: LC-MS chromatograms of TTX detection in the urine and blood samples spiked with 18 μmole of authentic TTX.
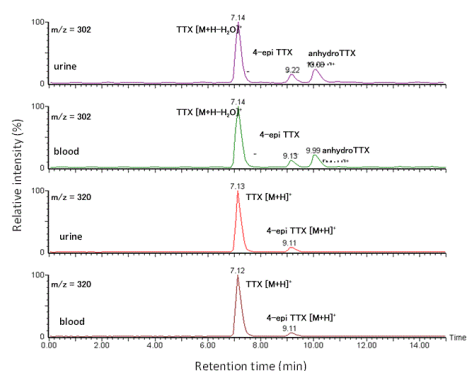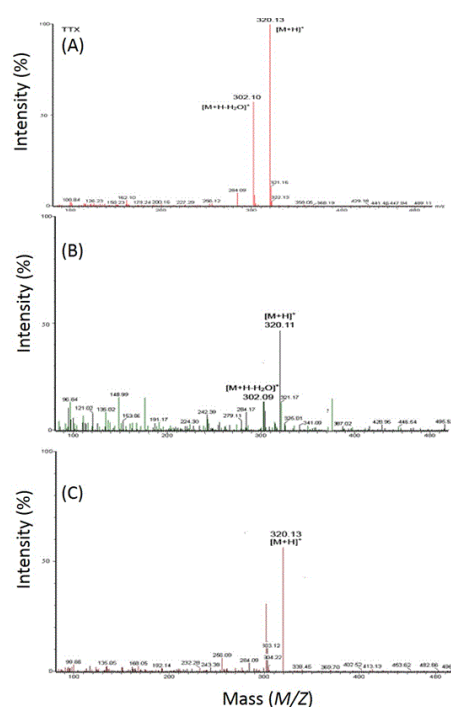

Figure 3: LC-MS chromatograms of the urine and blood of rat after orally treated with TTX toxin by using orogastric tube and then supported with saline solution for 4 hr.

Figure 4: The time course of TTX residue in the (A) blood and (B) urine of rat after orally treated with TTX toxin by using orogastric tube and then supported with saline solution and alkaline electrolyzed water.

Figure 5: LC-MS mass spectra of the urine (B) and blood (C) samples from rat, along with (A) authentic TTX.

It is not known whether chemical conversion of toxin in the body happens when the mouse assay is used.Therefore, LC-MS method provided the reliable and simple alternative for trace TTX toxin in the serum and urine. In addition, there are few reports in the literature of TTX concentrations being determined in poisoned patients. Kawatsu et al. [18] indicated urine samples from 12 TTX poisoned patients, values in the range 6ng/ml-100 ng/ml were found. Oda et al. [19] mentioned urine excretion values of 281 μg/day, for the second day after ingestion, dropping to 16 μg/day on day 4 and undetectable on day 5. Urine is the major excretion route of TTX intoxication for human beings [15]. The maximum peaks of TTX appeared at 6 hr in urine when the rats took distilled water. By the end of the collection period (60 hr), almost TTX was excreted in the urine.

Although alkaline electrolyzed water is not used for medical treatment so far, it could promote the secretion of TTX in rats in this study. Hence, alkaline electrolyzed water may be considered as a useful solvent to treat the TTX intoxicated patient for promoting the recovery.

The time course of TTX in the rat is more consistent with each other, suggesting that TTX is similarly redistributed from the serum to its target sites at sodium channels. Alkaline electrolyzed water has high pH value (9.0). This character may elevate the pH value of intestine and change TTX (pKa 8.76) and anh-TTX (pKa 7.95) to molecule type (not ionic type). The molecule type of TTX exhibits more lipid soluble.

Therefore, TTX could be quickly absorbed and entered the metabolism system [1,20].

Our results demonstrate that LC-MS method is capable of detecting small quantities of TTX in rat serum and urine samples. TTX was detectable up to 60 hr. Although the toxicolinetics of TTX elimination will differ for rats and humans, this study indicates that this approach could be useful for diagnosing or confirming a diagnosis of TTX in humans.

Acknowledgement

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