Short Communication

An Improved Tetrodotoxin Analyzer

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Previously, we constructed fluorometric tetrodotoxin (TTX) analyzers, by combining high-performance liquid chromatography (HPLC) and a post column reaction with hot NaOH solution.^{1,2)} These analyzers, although originally intended to replace conventional mouse bioassays, resulted in the discovery of TTX-producing bacteria³⁾ and the isolation of novel TTX analogs from newts⁴⁾ and puffers.^{5,6)} The previous models, however, were unsatisfactory for separating and detecting all the newly found TTX analogs. In this communication, we report an improved TTX analyzer for the sensitive determination of all TTX analogs.

Standard toxins of TTX, 4-*epi*TTX, 4,9-anhydroTTX, 6-*epi*TTX, 4,9-anhydro-6-*epi*TTX, 11-deoxyTTX, 4-*epi*-11-deoxyTTX and 4,9-anhydro-11-deoxyTTX, were prepared from newts,⁴⁾ and 11-*nor*TTX-6-(*R*)-ol from puffers.⁶⁾ The fluorescence spectra of TTX, 6-*epi*TTX, 11-*nor*TTX-6(*R*)-ol and 11-deoxyTTX were measured in 0.1N NaOH with a Hitachi 650-10S spectrometer, after incubating individual toxins with 2N NaOH in boiling water. The incubation times to give the highest fluorescence intensity were virtually the same for TTX, 6-*epi*TTX and 11-*nor*TTX-6(*R*)-ol (13~14 min), but were markedly different for 11-deoxyTTX (135 min). The maximum wavelengths of the excitation and emission spectra were also comparable among TTX (375, 500 nm), 6-*epi*TTX (375, 500 nm) and 11-*nor*TTX-6(*R*)-ol (375, 493 nm), but significantly

different for 11-deoxyTTX (412, 517 nm). The maximum fluorescence intensities of 6-*epi*TTX, 11-deoxyTTX and 11-*nor*TTX-6(*R*)-ol on a molar basis were approximately 28.5, 3.7 and 0.8 times that of TTX, respectively.

Separation of the analogs was performed on a Develosil ODS-5 column (1.0 × 25 cm) with 0.06N heptafluorobutyric acid in 0.001N ammonium acetate buffer (pH 5.0). The mobile phase was sent to the column with a Hitachi L-6200 pump at a flow rate of 1.0 ml/min. The eluate was mixed with 4N NaOH aqueous solution sent from another pump (1.1 ml/min). The mixture was passed through a stainless steel tube (0.5 mm × 2.5 m) immersed in a silicone oil bath, which was maintained at 135°C with a Thermo-minder H-100 regulator (Taiyo Scientific Industrial Co., Ltd.). A cooling jacket (30 cm) was placed between the reaction coil and the fluoromonitor (Hitachi F-1000, 12 μl cell), the excitation and emission wavelengths of the fluoromonitor being set at 375 and 500 nm, respectively. For the detection of 11-deoxyTTX and its analogs, another stainless coil (0.25 mm × 5 m) and a pressure gauge were attached to the outlet of the fluoromonitor to apply back pressure to prevent bubbling in the cell. The temperature of the silicone oil bath was raised to 180°C, and a line filter was placed at the end of the cooling jacket to trap solid particles produced from samples by the reaction. The excitation and emission wavelengths were set at 412 and 517 nm, respectively.

Chromatograms of the standard compounds are shown in Fig. 1A (analog except for the 11-deoxy group) and Fig. 1A' (the 11-deoxy group). Separation among 4,9-anhydro-6-*epi*TTX (c), 11-*nor*TTX-6(*R*)-ol (d) and 4-*epi*TTX (e) was insufficient; however, because of the low toxicity and low proportion of these three analogs in ordinary samples, this insufficient separation may not pose a problem in the routine monitoring of puffers. Figure 1B and B' show chromatograms of a sample solution prepared by the previous method¹⁾ from the liver of the puffer *Fugu niphobles*. The presence of 6-*epi*TTX (b) and 11-*nor*TTX-

Fig. 1. Chromatograms of a Mixture of Reference Toxins (A and A') and Sample Solutions Prepared from a Puffer Liver (B and B')⁶⁾ and a Newt Whole Body (C and C').⁴⁾

A: Mixture of TTX (a, 106 ng), 6-*epi*TTX (b, 8.53 ng), 4,9-anhydro-6-*epi*TTX (c, 12.6 ng), 11-*nor*TTX-6(*R*)-ol (d, 142 ng), 4-*epi*TTX (e, 119 ng) and 4,9-anhydroTTX (f, 55.7 ng).

A': Mixture of 11-deoxyTTX (g, 200 ng), 4-*epi*-11-deoxyTTX (h, 181 ng) and 4,9-anhydro-11-deoxyTTX (i, 181 ng).

B and B': Injected amounts were equivalent to 5 mg and 10 mg of puffer (*Fugu niphobes*) liver, respectively. C and C': Injected amounts were equivalent to 5 mg and 10 mg of the newt (*Cynops ensicauda*) whole body, respectively.¹⁾

The reaction temperature, and the excitation and emission wavelengths of the fluoromonitor were set at 135°C, and 375 and 500 nm for A, B and C; and at 180°C, and 412 and 517 nm for A', B' and C'. The range of the fluoromonitor was set at 10, except for C (range 1).

6(*R*)-ol (d), which were undetectable with the previous model could be clearly observed under the present conditions (Fig. 1B). 11-deoxyTTX and its analogs were below the detection level (2.0 ppm) in this species (Fig. 1B'). A similar extract prepared from the whole body of the newt *Cynops ensicauda*

had a significantly different toxin profile. Prominent peaks in the chromatogram (Fig. 1C) are those of 6-*epi*TTX (b) and 4,9-anhydro-6-*epi*TTX (c), and at the elevated reaction temperature, 11-deoxyTTX (g) was detectable (Fig. 1C').

The dose-fluorescence response was prepar-

ed for TTX, 6-*epi*TTX, 11-deoxyTTX and 11-*nor*TTX-6-(*R*)-ol, measurements being triplicated, and the mean value of peak heights was plotted against the injected amount of each toxin. Good linearity was observed in the range of 9.6 ng ~ 9.45 μ g for TTX, 1.2 ng ~ 1.89 μ g for 6-*epi*TTX, 20.4 ng ~ 9.45 μ g for 11-deoxyTTX, and 12.6 ng ~ 0.2 μ g for 11-*nor*TTX-6-(*R*)-ol ($S/N > 3$). The dose-response relationships can be expressed by the equation $Y = 2.08X - 0.02$ ($r = 0.999$) for TTX, $Y = 16.7X - 0.04$ ($r = 0.998$) for 6-*epi*TTX, and $Y = 0.998X + 0.02$ ($r = 0.999$) for 11-deoxyTTX, where Y stands for the peak height in cm in the range 0.2 of the fluoromonitor, and X the amount of each toxin in μ g. The regression line for 11-*nor*TTX-6-(*R*)-ol can be expressed by the equation $Y = 79.2X + 0.04$ ($r = 0.999$), where Y stands for the peak height in cm in the range 10 of the fluoromonitor.

The new TTX analyzer described here allowed us to analyze all the TTX analogs known before, and may be useful for exploring new TTX analogs in various biota. Because many TTX precursors and metabolites are predictably much less toxic than TTX and thus difficult to be monitored by biological tests,

the analyzer will be an effective tool to investigate the metabolic pathways of this extremely interesting toxin. The separation between TTX and 6-*epi*TTX achieved in the present study represents important progress for improving the accuracy of the analyzer, as the two components differ very much in their dose-response relationships. The new analyzer was especially useful in monitoring tropical species, which contained 6-*epi*TTX in relatively high concentrations.

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