Parameters of tetrodotoxin (TTX) and its analogues analysis

Tetrodotoxin and its analogues (TTXs) were identified using HPLC–MS/MS. The HPLC system included two pairs of LC-30 pumps, a SIL-30AC autosampler, a CTO-20A thermostat, an SCL-20A system controller, and a triple quadrupole mass spectrometer LCMS-8060 (Shimadzu Europa, Duisburg, Germany) with electrostatic spray ionization (ESI). Separation was carried out using a SeQuant ZIC HILIC column (150 × 2.1 mm, 5 μm) (Merck, Darmstadt, Germany) at 40 °C and a flow rate of 0.25 ml/min. A binary gradient was used: mobile phase A, ammonia (5 mM) and formic acid (8 mM) in 9:1 acetonitrile–water; mobile phase B, ammonia (10 mM) and formic acid (20 mM) in water. A gradient profile was used as follows: (a) 0–4.3 min, 15% B; (b) 4.4–17 min, 25% B; (c) 17–20 min, 50% B. The sample volume was 1 μL. A SeQuant ZIC-HILIC guard column (20 × 2.1 mm, 5 μm) (Merck, Darmstadt, Germany) was installed in line before the analytical column through a two-position 6-port valve. At 4.4 min, the valve was switched and guard columns backflashed with isopropanol (4.4–7 min) and water (7–14 min) at flow rate of 0.2 ml/min. At 17 min, the valve was switched back. The mass spectrometer was operated in scan (m/z 200–1000) and multiple reaction monitoring (MRM) modes. The ion source parameters were as follows: interface temperature—280°C, desolvation line temperature—250°C, nebulizing gas (N2) flow—3 L/min, drying gas (N2) flow—3 L/min, and heating gas (dry air) flow—17 L/min. The TTX concentration was calculated using the calibration curve of a standard TTX (purity > 99%) solution series (Cat No: T-550, Alomone Labs Ltd., Jerusalem, Israel). The toxin detection criteria included a precursor MRM transition peak S/N ratio > 3, relative intensity of the fragment ion peak > 4%. MRM transitions, collision energy and the order of toxins elution (retention time) were used to detect TTX analogues as described by Bane et al. (2014)[42], Shoji et al. (2001)[43], Vale (2008)[44], Kudo et al. (2012)[45], Yotsu-Yamashita et al. (2013)[46], and Turner et al. (2017)[47] and indicated in Table 1. The concentrations of TTX analogues were calculated following the procedure of Chen et al. (2011)[48]. The method was validated using standard TTX solutions in MRM mode. The linearity range was from 0.0006 to 0.1 μg/g. The recovery range from 0.001 to 0.1 μg/g of TTX was 98.4%. The limit of quantification (LoQ) was 0.0006 μg/g (0.6 ng/mL). The limit of detection (LoD) was 0.0002 μg/g (0.2 ng/mL), and the relative SD was 4.5%–14.6%.

Table 1 MRM transitions, collision energy and retention time for TTX and its analogues.

№	Analogue	MRM transitions	Collision Energy (eV)	Retention time (min)
1	TTX	320.1>302.1	25, 38	12.48
		320.1>162.1	36.949–55.423	

2	4-epiTTX	320.1>302.1 320.1>162.1	25, 38	11.51
3	5,6,11-trideoxyTTX	272.1>162.1 272.1>254.1 208.1064; 180.1137; 178.0954; 162.1020; 133.0724;	33.570–50.358	5.64
4	4,9-anhydroTTX	302.1>256.1 302.1>162.1	30 49	10.32
5	11-oxoTTX	336>318 336>300 336>282 336>178 336>162	38.1–57.1	13.9
6	4-epi-11-oxoTTX	320.1>302.1 320.1>162.1	25–40	13.37
7	5-deoxyTTX/11-deoxyTTX*	304>286 304>176	49	9.8 9.21
8	11-norTTX-6(R)-ol /11-norTTX-6(S)-ol*	290/272 290.1>162.1	49 30	10.33, 11.39
9	6,11-dideoxyTTX	288>224	34.6831–52.046	8.18

* toxins are not differentiated.

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