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Short communication

Presence of norepinephrine and other biogenic amines in stonefish venom

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Abstract

Although fish venoms exert a cardiovascular effect, the presence of adrenergic substances was not previously demonstrated. Chromatographic analysis with electrochemical detection showed the presence of substances co-migrating with norepinephrine, dopamine and tryptophan. Serotonin, which was thought to be implicated in the intense pain following fish envenomation, was not detected. Norepinephrine was identified as a component of the stonefish *Synanceia verrucosa* venom by gas chromatography–mass spectrometry.

Keywords: Norepinephrine; Dopamine; Tryptophan

1. Introduction

The most common symptom after injection of fish venom is an immediate, intense sharp pain. This reaction may be accompanied by headache, nausea or vomiting, cardiac palpitation, tachycardia or bradycardia and, as we observed, pulmonary oedema. Carlisle [1] has reported that the dialysable fraction of weeverfish venom (*Echiichtys* (=*Trachinus*) *vipera*) contains serotonin and a substance acting as an histamine releaser, the non-dialysable fraction being responsible for the lethal effect. In fact, the presence of serotonin has yet to be demonstrated in fish venom, although many authors have repeated Carlisle's assertion. Our group and others have failed to detect serotonin by histochemical procedures or fluorescence technique [2,3].

Acetylcholine, epinephrine (E) and norepinephrine (NE) are the best known regulators of cardiac physiology and arterial pressure, and they have long been implicated in the toxic effects of fish venoms (the scorpionfish *Scorpaena guttata* [4,5]; the lionfish *Pterois volitans* [6]; the stonefish *Synanceia* [7,8]) or in crinotoxins (the pardaxin from *Pardachirus marmoratus* [9,10]; *Arius thalassinus* [11]). Venom may contain acetylcholine [4,10] or induce a massive release of neurotransmitters such as acetylcholine (*Pterois volitans* [6] *Synanceia trachynis* [7,8] from nerve terminals. On the other hand, *Scorpaena guttata* venom, which induces a primary muscarinic action, has also a secondary β-adrenergic action [4,5]. Injection of a low dose of *Synanceia*

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venom is followed by a transient hypertension [12–14], apparently stemming from the action of substance(s) present in both fresh and frozen stored venom, which induce(s) a positive inotropic response [15,16]. We have recently shown that fresh samples of venom from *Synanceia verrucosa* exert a positive inotropic effect on the frog heart preparation; this effect being inhibited by propranolol, but not by yohimbine or urapidil [15,16]. This suggests either the presence of an epinephrine-like substance in the venom, or its release from the preparation. The present experiments were designed to find out whether stonefish venom contains a β-adrenergic agonist.

2. Experimental

2.1. Venom

Stonefish *(Synanceia verrucosa* and *Synanceia horrida)* originally from the Philippines were maintained in the laboratory. Venom was sampled with a syringe from anaesthetized fish (phenoxyethanol 0.04% in sea water, Romeil), then immediately used or stored at −20°C until analysis by high-performance liquid chromatography linked to electrochemical detection (HPLC–ED) or gas chromatography coupled with mass spectrometry (GC–MS).

2.2. HPLC with electrochemical detection (HPLC–ED)

The chromatographic system consisted of a Beckman 116 pump (constant flow-rate 0.9 ml/min), a Rheodyne injection valve (Touzart and Matignon, Les Ulis, France; 20-μl loop), a reversed-phase column Kromasil C_{18} (T.C. 19%, 5 μm, 250×4.6 mm, Touzart and Matignon) and an electrochemical detection cell 656 Metrohm ELCD (Herisau, Switzerland) fitted with a glassy carbon electrode and an Ag/AgCl reference electrode connected to a Metrohm 641 VA detector. The detector potential was set at 0.88 V and the sensitivity for a full scale detection of 1 nA. A mixture in double distilled, deionized water of phosphate buffer (100 m*M* potassium KH_2PO_4; Merck–Clévenot, Nogent-sur-Marne, France), 0.1 m*M* EDTA disodium salt (Titriplex III), 5 m*M* sodium heptane sulphonate (Pic B7, Waters) and 7.5% (v/v) methanol (HPLC grade, Fisons, Loughborough, UK) was used as the mobile phase (pH 3.76).

All procedures were carried out at 4°C. After venom dilution in phosphate buffer (10 m*M*, NaCl 50 m*M*, pH 7.8) and centrifugation (45 000 *g*, Sorvall RC5B, Dupont Instrument), the supernatant was adjusted to 1 mg protein per ml with buffer, constituting the crude venom. Crude venom samples were homogenised (v/v) in 0.4 *M* $HClO_4$ solution containing antioxidants (w/v: 0.1% cysteine; 0.1% $Na_2S_2O_5$; 0.1% Na_2EDTA) to precipitate proteins. Following centrifugation (9000 *g*; Microfuge Beckman, 5 min), the clear supernatants were removed and after membrane filtration (0.22 μm pore size, Millipore, Bedford, MA, USA) subjected to HPLC–ED.

The quantitative data were obtained from either two (*S. horrida*) or three (*S. verrucosa*) venom samples (fresh or frozen) taken from different dorsal fins. Each value corresponded to at least five different analyses of the same sample. The results were expressed as μg of biogenic amine per milligram of protein.

2.3. Gas chromatography–mass spectrometry

For gas chromatography–mass spectrometry, the biological samples and reference substances were derivatized [17] to increase the volatility of the compounds.

Venom was homogenised in 0.4 *M* $HClO_4$ (1 mg protein/ml), centrifuged (9000 *g*; 5 min), and filtered (0.22 μm pore size). The samples and NE (Sigma) (1 mg NE/ml) used as reference substance were treated at the same time under identical conditions.

2.3.1. Derivatization of compounds

After evaporation by heating, the residues were dissolved in dichloromethane and immediately evaporated under a stream of nitrogen. Toluene (20 μl) and N-methyl-N-trimethyl silyltrifluoroacetamide (60 μl) (Fluka, Buchs, Switzerland) were added to the extract and the solution was incubated for 1 h at 70°C.

2.3.2. GC separation

GC separation was performed on a WCOT fused-silica column with a stationary phase CP-Sil-5CB (length 50 m; 0.32 mm I.D.; 0.45 mm O.D.). The temperature program was 1 min at 100°C, ramped to 170°C at 30°/min to 210°C at 4°C/min and to 280°C at 10°C/min. The carrier gas was helium at a flow-rate of 1 ml/min. Samples (1 μl) of a solution at 1 mg/ml were injected. The injector temperature was 150°C. The interface temperature was 275°C.

2.3.3. MS analysis

MS analysis was performed on a triple quadrupole GC–MS–MS system (Nermag R-30-10) tuned with FC43 calibration compound. The sample was analysed under positive chemical ionisation with ammonia. The ammonia pressure introduced in high pressure source either through GC mode or by using direct solid probe. The ion source temperature was lower 100°C and the pressure was $0.8 \cdot 10^{2}$ Pa and the secondary vacuum was $1.73 \cdot 10^{-2}$ Pa. The repeller was adjusted at 0 V and the electric filament at 100 μA. Each spectrum corresponds to the average of 50 scans.

3. Results and discussion

An HPLC-ED chromatogram of standard compounds is shown in Fig. 1. For comparison, the chromatograms from *S. horrida* and *S. verrucosa* venoms are shown in Fig. 2 and Fig. 3. The assay sensitivity and the retention times are listed in the legend to Fig. 1.

Compounds were co-migrating with NE, dopamine (DA) and tryptophan (Trp). *S. horrida* venom was found to contain about 1.2 μg NE, 2.37 μg DA and 0.54 μg Trp/mg protein. For *S. verrucosa*, the same compounds were characterised but, although DA and Trp were less concentrated in this venom

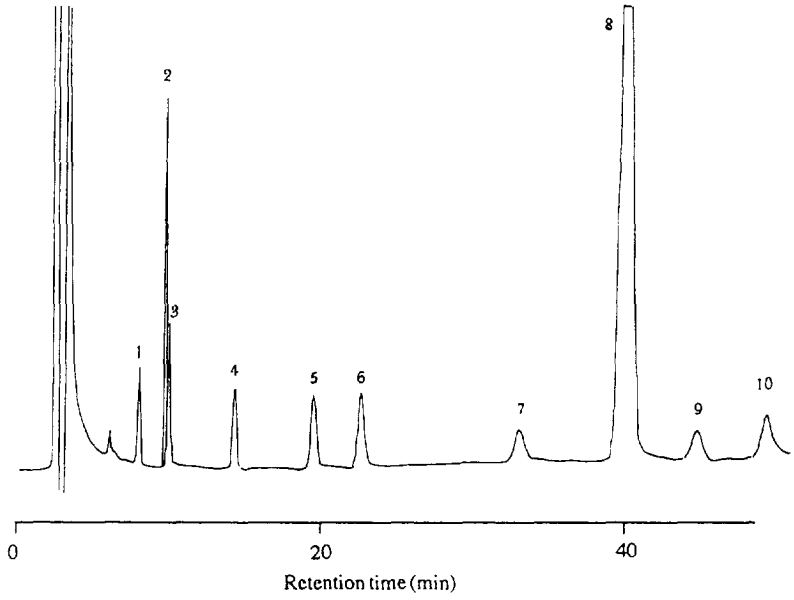

Fig. 1. HPLC–ED chromatogram of standard biogenic amines. For standards (all from Sigma; injection 0.4 ng, except Trp, 8 ng and epinephrine, 2 ng), retention time and detection limit in biological samples are in brackets. 1=Norepinephrine (NE, 8.13 min, $5.10 \cdot 10^{-4}$ μg/mg protein), 2=Epinephrine (E, 9.98 min, $1.70 \cdot 10^{-3}$ μg/mg protein) 3=5-hydroxytryptophan (5-HTP, 10.00 min, $9.93 \cdot 10^{-4}$ μg/mg protein), 4=Dihydroxyphenylacetic acid (DOPAC, 14.68 min, $1.60 \cdot 10^{-3}$ μg/mg protein), 5=Dopamine (DA, 20.13 min, $5.26 \cdot 10^{-4}$ μg/mg protein), 6=5-hydroxyindoleacetic acid (5-HIAA, 23.41 min, $6.59 \cdot 10^{-4}$ μg/mg protein), 7=Homovanillic acid (HVA, 34.66 min, $1.62 \cdot 10^{-3}$ μg/mg protein), 8=Tryptophan (Trp, 40.66 min, $3.93 \cdot 10^{-3}$ μg/mg protein), 9=3-methoxytyramine (3-MT, 46.35 min, $1.74 \cdot 10^{-3}$ μg/mg protein) and 10=Serotonin (5-HT, 50.96 min; $1.15 \cdot 10^{-3}$ μg/mg protein).

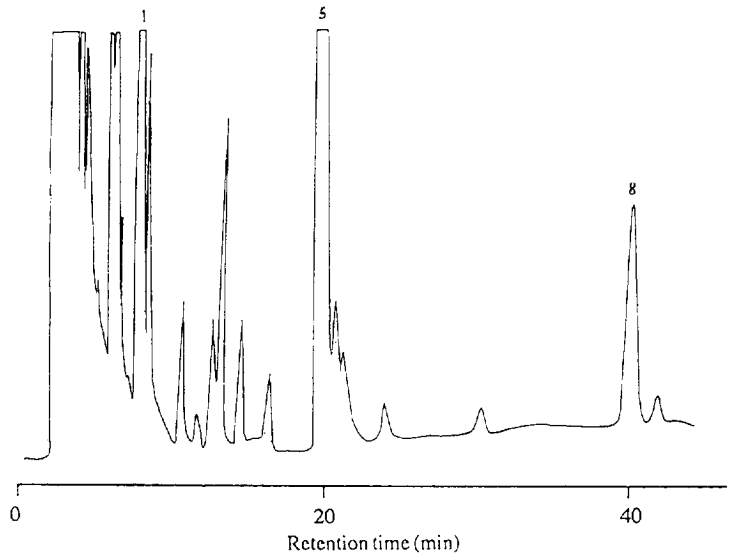

Fig. 2. Typical chromatogram of a sample from *Synanceia horrida* containing 6.5 μg of protein. The major peak corresponds to DA. 1=NE; 5=DA; 8=Trp.

(respectively 0.03 and 0.16 μg/mg protein), it contained correspondingly more NE (8 μg/mg protein) than the *S. horrida* venom. Storage at −20°C did not seem to alter the concentrations. 5-HT was consistently below the detection threshold ($1.15 \cdot 10^{-3}$ μg/mg protein) of our system.

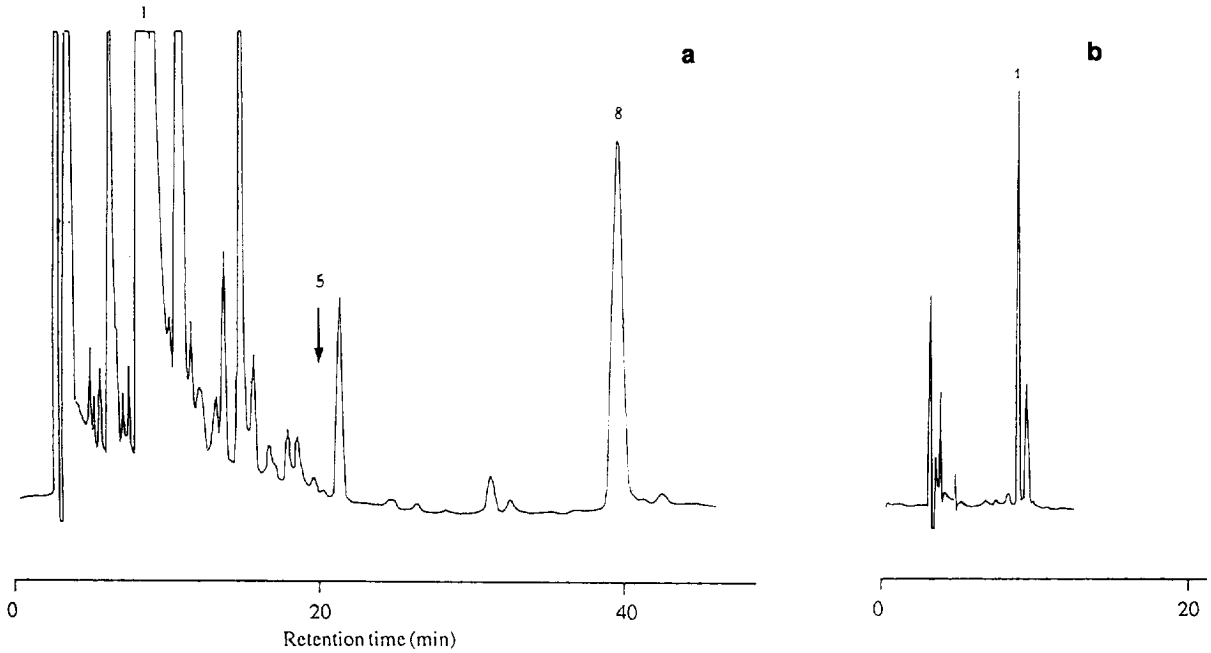

Fig. 3. Typical chromatogram of a sample from *Synanceia verrucosa* containing 20 μg (a) or 0.3 μg of protein allowing NE peak to be resolved (b). The major peak corresponds to NE. 1=NE; 5=DA; 8=Trp.

S. verrucosa venom was also subjected to GC–MS. The GC peak of the NE reference had a retention time of 13 min 43 s. NE mass spectrum (Fig. 4a) recorded under chemical ionisation displayed four major peaks (*m/z* 296, 313, 355 and 386). The protonated MH^+ molecules appeared at *m/z* 386. This molecular species corresponded to the derivatized norepinephrine (Fig. 5). Under the same analytical conditions, venom presented one minor peak with a retention time of 13 min 20 s corresponding to NE. Its spectrum (Fig. 4b) was identical to that of the reference. In each case, for both venom or standard, the base peak represented the ion MH^+ (*m/z* 386). The relative abundance of the different ions: $MH-HOSi(CH_3)_3^+$ (*m/z* 296), $MH-HOSi(CH_3)_3+NH_3^+$ (*m/z* 313), $MH-CH_3NH_2^+$ (*m/z* 355) and MH^+ (*m/z* 386) was approximately identical to those observed from the standard and the venom sample. These fragment ions enabled confirmation of the structure. Indeed, the presence of the diagnostic ions was consistent with norepinephrine sample: (i) the benzylic cleavage was the most favorable process (i.e. *m/z* 296 was the base peak of fragment ions), (ii) this one could be solvated in gas phase by ammonia to give rise to formation of *m/z* 313, (iii) a second possible benzylic rupture concerning the methylamine loss (*m/z* 355) was observed. Note that from the latter species, ion solvation induced by NH_2 did not take place very freely, because of the presence of $O\text{-}Si(CH_3)_3$ group which stabilized the positive charge. Finally, absence of MNH_4^+ was expected, since the proton affinity of this substrate was higher than that of ammonia.

The finding that NE is present in stonefish venom has implications for venom activity. For instance, at a dose of 0.7 to 2.9 μg protein/ml, venom has a positive inotropic and chronotropic action [15,16]. According to the present data, this is compatible with

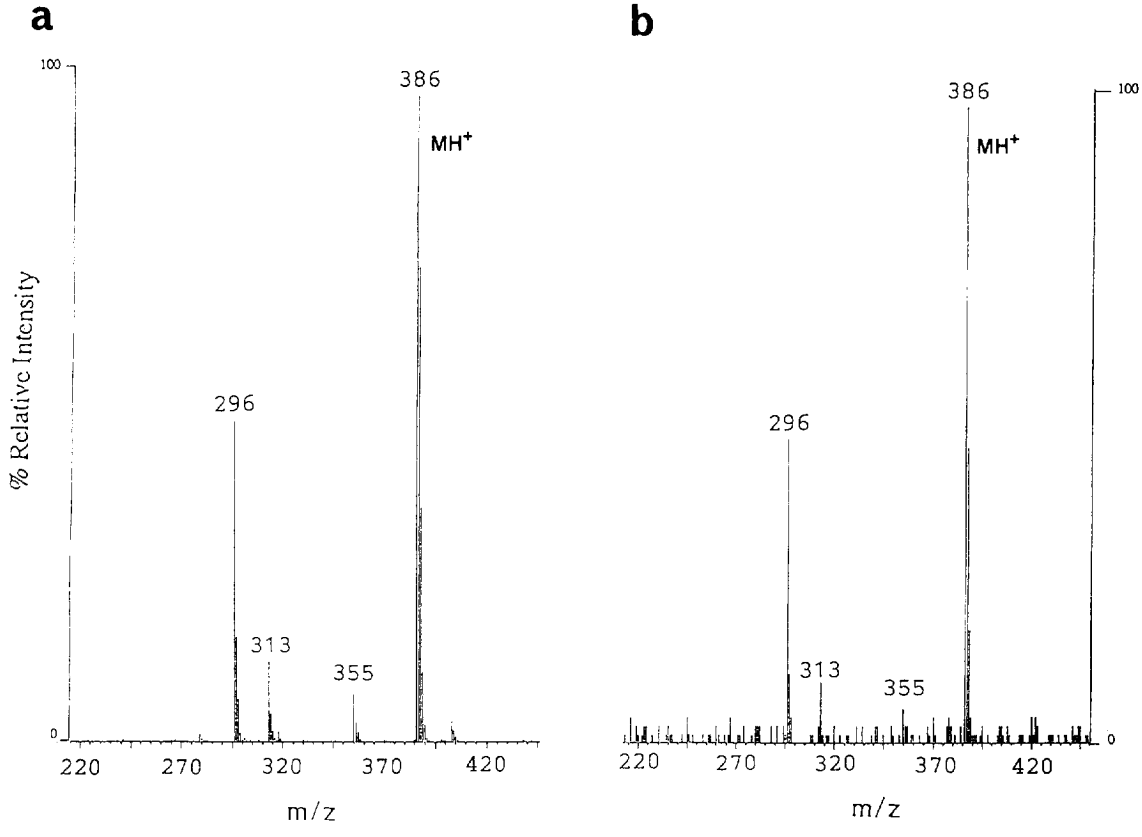

Fig. 4. Mass spectra of norepinephrine (a) and sample venom (b).

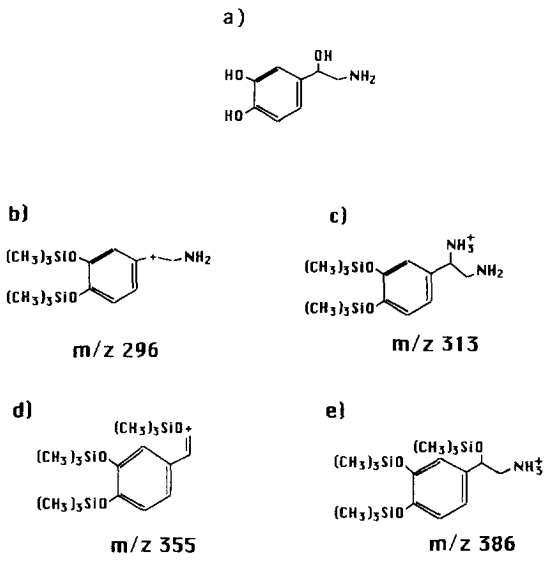

Fig. 5. Structure of: (a) Norepinephrine (M_r = 169), (b–e) different ions and their corresponding m/z obtained after GC–MS.

levels of $3.3 \cdot 10^{-8}$ to $1.4 \cdot 10^{-7}$ M of NE in the venom. Since the lethal proteins are highly labile [18–20], even when stored at −80°C, the proportion of NE in the pharmacologically active compounds may increase with time in storage. This should be taken into account in investigations on the cardiovascular actions of venom.

Acknowledgments

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