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Distribution of ^{125}I-labeled crotamine in mice tissues

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Abstract

Crotamine is a strong basic polypeptide from *Crotalus durissus terrificus* (*Cdt*) venom composed of 42 amino acid residues tightly bound by three disulfide bonds. It causes skeletal muscle spasms leading to spastic paralysis of hind limbs in mice. The objective of this paper was to study the distribution of crotamine injected intraperitoneally (ip) in mice. Crotamine was purified from *Cdt* venom by gel filtration followed by ion exchange chromatography, using a fast-performance liquid chromatography (FPLC) system. Purified crotamine was irradiated at 2 kGy in order to detoxify. Both native and irradiated proteins were labeled with ^{125}I using chloramine T method, and separated by gel filtration. Male Swiss mice were injected ip with 0.1 mL (2×10^6 cpm/mouse) of ^{125}I native or irradiated crotamine. At various time intervals, the animals were killed by ether inhalation and blood, spleen, liver, kidneys, brain, lungs, heart, and skeletal muscle were collected in order to determine the radioactivity content. The highest levels of radioactivity were found in the kidneys and the liver, and the lowest in the brain.

Keywords: Crotamine; Toxin; Biodistribution; *Crotalus*; Venom; Gamma radiation

1. Introduction

Snake bites represent a major public health problem, particularly in subtropical countries. In Brazil, the genus *Crotalus* holds a special significance and still is the cause for high rates of animal envenomation and mortality (Brazilian Health Ministry—Ministério da Saúde do Brasil, 1986).

Crotamine is a strong basic polypeptide (pI 10.3) from *Crotalus durissus terrificus* (*Cdt*) venom composed of 42 amino acid residues tightly bound by three disulfide bonds (Laure, 1975). Nicastro et al. (2003) solved the structure of crotamine in solution that comprises a short N-terminal α-helix and a small antiparallel triple-stranded β-sheet arranged in a $\alpha\beta1\beta2\beta3$ topology never before encountered among active toxins on ion channels. It produces skeletal muscle spasms leading to spastic paralysis in the hind limbs in mice (Cheymol et al., 1971; Chang and Tseng, 1978; Vital-Brazil

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et al., 1979), Pellegrini Filho et al. (1978) and Matavel et al. (1998) proposed the action of crotamine on Na^+ channels.

Crotamine induces spasticity and convulsions in mice and rats when injected directly in the brain (Habermann and Cheng-Raude, 1975). However, its primary mechanism of action and its role in crotalic envenomation have not been entirely clarified.

The treatment for crotalic accidents is sorotherapy. Several studies have been performed in order to attenuate venom toxicity. Among the methodologies applied, ionizing radiation has proved to be a powerful agent. Ionizing radiation can change the molecular structure and modify the biological activity of proteins and peptides affecting the biological properties of biomolecules. These properties have been successfully employed to attenuate animal toxins (Hati et al., 1990; Nascimento et al., 1996). Our group has shown that the gamma radiation reduced the toxicity of crotamine to about two fold (Boni-Mitake et al., 2001).

Despite several studies on the pharmacological effects of crotamine in the literature, the biodistribution study of this toxin has not been reported yet. In most of the reports concerning pharmacokinetics of toxins, the experiments were performed using the total venom. Snake venoms are composed of many proteins made up of different structures and molecular weights with different toxicological and physiopathological effects, which are sometimes synergistic. This makes it very difficult to follow each of the components in a pharmacokinetics study when working with the total venom. In the present paper, we report the biodistribution study of crotamine purified from the total *Cdt* venom. We also compared the biodistribution of native and gamma-irradiated crotamine.

2. Materials and methods

2.1. Venom, animals and chemicals

Crude air-dried venom from the South American rattlesnake, *Cdt* was supplied by the Butantan Institute (São Paulo, Brazil). Mice used in the experiment were obtained from the colony housed at IPEN/CNEN (São Paulo, Brazil). They were maintained under a 12L:12D cycle (lights on at 07:00 am) in temperature-controlled environment ($22 \pm 2\,^{\circ}C$). Food and water were freely available. Salts and other chemicals used in this study were of the best quality available (ACS grade).

2.2. Purification and irradiation of crotamine

Crotamine was isolated and purified from the venom of the *Cdt* snake and was irradiated as described by Boni-Mitake et al. (2001). The purity of crotamine was confirmed by the N-terminal analysis of five residues: Tyr–Lys–Gln–Cys–Hys and by amino acid analysis. The amino acid composition of the native toxin was the same as previously reported (Laure, 1975), and no alterations in composition were observed in irradiated crotamine (Boni-Mitake et al., 2001).

2.3. Radioiodination of crotamine

Native (Nc) or irradiated (Ic) crotamine was labeled using 29.6 MBq of ^{125}I by chloramine T method (Hunter and Greenwood, 1962).^{125}I-labeled crotamine was purified by Sephadex G-50 (Pharmacia) column (2.5×47 cm) using 50 mM sodium phosphate buffer, pH 7.4, as eluent.

2.4. Body distribution of ^{125}I-labeled crotamine

A 100 μl aliquot of ^{125}I-labeled native crotamine (^{125}I-Nc) (2.6×10^6 cpm) or 300 μl aliquot of ^{125}I-labeled irradiated crotamine (^{125}I-Ic) (1.3×10^6 cpm) was intraperitoneally (ip) injected in Swiss mice (35 ± 2 g). Blood samples were obtained from the retro-orbital vessels at appropriate time intervals and analyzed for radioactivity using a well-type gamma scintillation counter (Oakfield Instruments Ltd.). At various time intervals, mice were killed by ether inhalation and the organs (liver, spleen, lungs, kidneys, heart, brain, leg muscle) were dissected. Afterwards, they were weighed and their radioactivity measured in a well-type gamma scintillation counter (Oakfield Instruments Ltd). Each individual sample was analyzed until it accumulated 1000 counts. Results were expressed as cpm/organ. It was difficult to measure the radioactivity accumulated in the matrix of organs owing to the fact that it also contained the radioactivity attributable to the blood residing in the organs, in addition to the organ matrix itself. To overcome this difficulty, we calculated the net radioactivity of each organ by subtracting the radioactivity corresponding to the blood in the organ from that of the whole organ. The blood volume of each organ was assumed to be as reported by Altman (1961).

2.5. ^{127}I labeling of crotamine

^{127}I labeling of Nc (1.2 mg) was performed by chloramine T method (Hunter and Greenwood, 1962). ^{127}I-labeled crotamine was diluted in 50 mM sodium phosphate buffer, pH 7.4 to a final protein concentration of 0.9 mg/mL. As control a similar labeling to the procedure, as described above, was performed, although without the presence of crotamine.

2.6. Bioactivity test of iodinated native crotamine

Swiss mice (30 ± 2 g) were distributed into three groups, with five animals each. The animals received 6.0 mg/kg (LD_{50}) of Nc or ^{127}I-labeled crotamine via ip. The control group received labeling solution. The time to produce the characteristic paralytic signs of hyperextension of hind limbs associated with crotamine was observed.

2.7. Statistical analysis

All data were expressed as mean $\pm$SD. Statistical comparisons of various groups and time intervals were performed using the *F*-test. The blood concentration curves of Nc and Ic related to biodistribution study were fitted by non-linear regression based on Levenberg–Marquardt algorithm (Microcal Origin, version5.0). Differences were considered significant at $p < 0.05$.

3. Results

3.1. Radioiodination ^{125}I of crotamine

Total radioactivity after purification of Nc and Ic was 53.5% and 68.7%, respectively. The labeling efficiency was 47.0% to Nc and 15% to Ic.

3.2. Radioiodinated crotamine purification

The elution profiles of ^{125}I-Nc and ^{125}I-Ic on a Sephadex G-50 column are represented by Fig. 1. The radioactivity and protein peaks coincide in both conditions.

3.3. Iodinated crotamine bioactivity

The results show that 80% of the mice injected with crotamine labeled with ^{127}I had spastic paralysis of hind limbs six minutes after the injection and 25% of the mice died after 48 h.

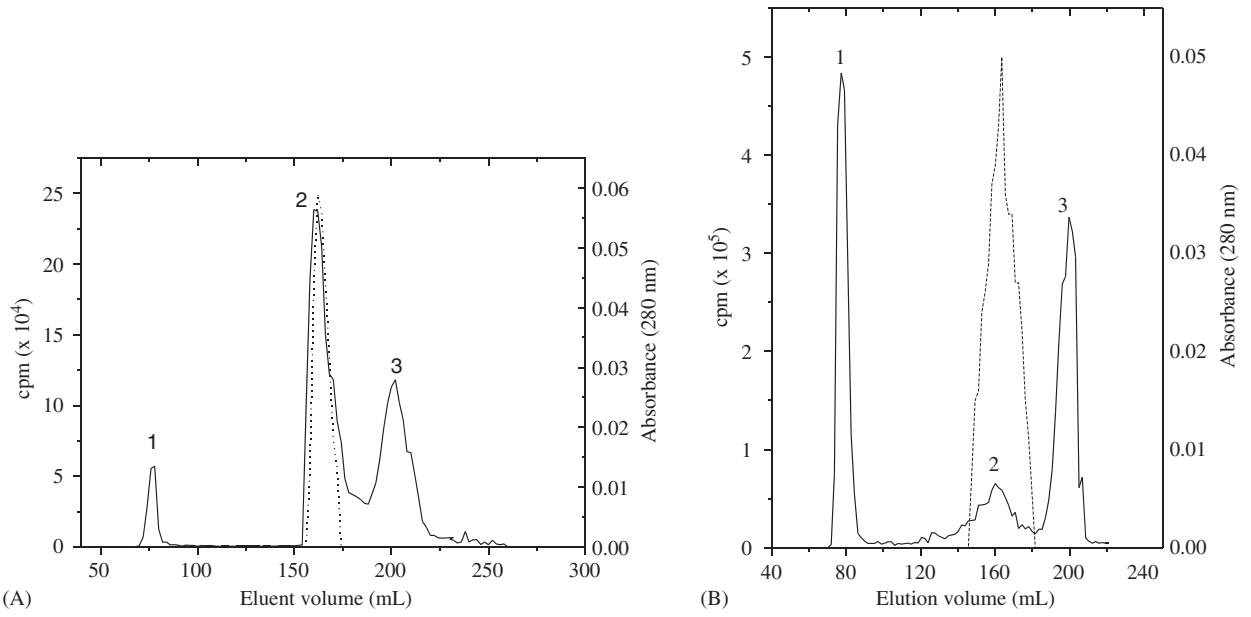

Fig. 1. (A) Size exclusion chromatography of ^{125}I-Nc (—) and Nc (–) in 50 mM sodium phosphate, pH 7.4, on a Sephadex G-50 (2.5×47 cm). Flow rate: 0.2 mL/min. The complete procedure was performed at 4 °C. Peaks: 1. blue dextran, 2. radiodinated crotamine, 3. Free ^{125}I. Dashed line represents Nc peak. (B) Size exclusion chromatography of ^{125}I-Ic (—) and Ic (–) in 50 mM sodium phosphate, pH 7.4, on Sephadex G-50 (2.5×47 cm) column. Flow rate: 0.2 mL/min. Temperature: 4 °C. The complete procedure was performed at 4 °C. Peaks: 1, blue dextran; 2, ^{125}I-Ic; 3, free ^{125}I. Dashed line represents Ic peak.

3.4. Native and irradiated crotamine biodistribution

The fitted curves correspond to a linear combination of two exponential terms following the equation below:

$$Cs(t) = A_1 \times e^{-b_1 \times t} + A_2 \times e^{-b_2 \times t},$$

where Cs is the blood concentration at a given time (t), A_1 and A_2 are respective intercepts of the initial and final phases, and the coefficients b_1 and b_2 were derived graphically from the slopes of the biphasic curves to primer order kinetic process, as shown in the equation below (Cid, 1982):

$$t_{máx} = \frac{\ln\, b_2/b_1}{b_2 - b_1}.$$

The parameter values A_1 and A_2, and the exponential coefficients b_1 and b_2 to Nc and Ic, were calculated by the software Microcal Origin (version 5.0) and are shown in Table 1.

Table 1
Parameter values of fitted blood concentration of ^{125}I-Nc and ^{125}I-Ic

Parameter	Native		Irradiated	
	CV	SD	CV	SD
A_1	36,593	5143	59,844	37,873
b_1	0.102	0.032	0.28	0.12
A_2	−29,601	9436	−41,805	36,567
b_2	3.6	2.4	0.98	0.71

CV: calculated value, SD: standard deviation.

F-test was performed on the fitted curves to Nc and Ic, demonstrating that there was no difference between the experimental and the calculated data.

The Nc and Ic were rapidly absorbed; the maximum blood concentration (t_{max}) was observed in 1 h for Nc and 1.8 h for Ic (Fig. 2).

Liver, kidneys and skeletal muscle had the highest amounts of Nc and Ic at all time intervals; however, lungs and spleen had the smallest amounts of crotamine. There was statistical significance between Nc and Ic only for liver and kidneys (Table 2). The Nc and Ic were not detected in the brain. The highest tissue amounts of Nc and Ic were detected at around 1 h after injection.

4. Discussion

The crotamine is a protein composed of 42 amino acid residues, with only one tyrosine residue in the N-terminal (Laure, 1975). The labeling of crotamine with ^{125}I could interfere in its activity, considering that the labeling method is based on iodination of the tyrosine residue of this toxin (Hunter and Greenwood, 1962). Literature data suggest that the iodination of tyrosine abolishes the toxicity of toxin E, which is homologue of crotamine (Allen et al., 1996). Thus, in the present work, 6 mg/kg (LD_{50}) of ^{127}I-labeled crotamine was injected in mice, and the results showed that 80% of them had hind limbs paralysis and 25% of the animals died in 48 h, indicating that iodination of tyrosine N-terminal did not compromise the crotamine biological activity.

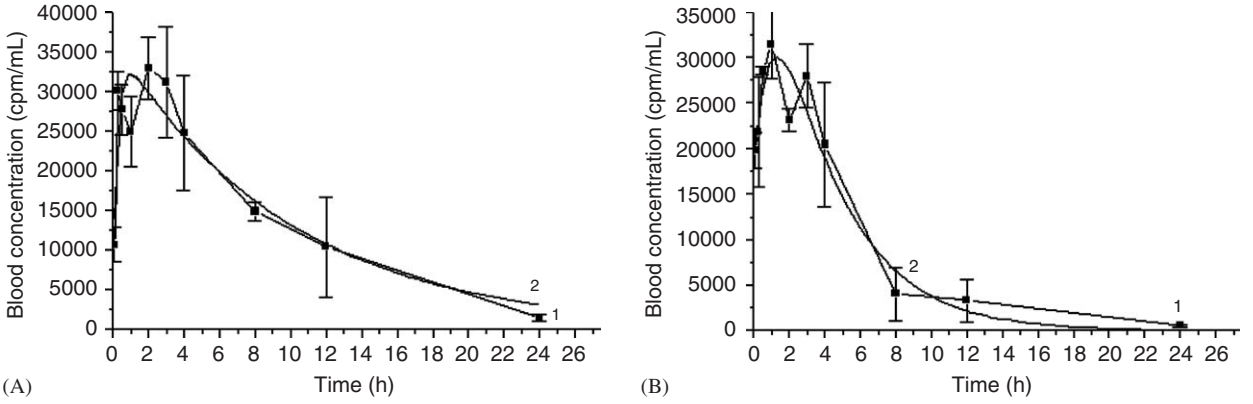

Fig. 2. (A) Blood concentration of ^{125}I-Nc at different time intervals, after ip injection (line 1). The data represent the mean of 4–5 mice. Line 2 is the fitted curve. (B) Blood concentration of ^{125}I-Ic at different time intervals, after ip injection (line 1). The data represent the mean of 4–5 mice. Line 2 is the fitted curve.

Table 2
Tissue distribution of ^{125}I-Nc and ^{125}I-Ic at different time intervals after ip injection

Tissue	Time (h)					
		0.25	1	4	8	24
Liver	NC[a]	5.2±1.5	10.0±4.0	5.7±2.2	8.8±2.2	1.3±0.5
	IC	1.9±0.3	2.4±0.5	2.3±0.3	1.2±0.3	0.49±0.0.7
Kidneys	NC[a]	3.3±0.7	4.8±1.6	3.6±1.0	1.1±0.2	0.24±0.06
	IC	4.9±0.4	15.1±2.3	11.7±1.3	7.3±0.5	2.82±0.08
Skeletal muscle	NC	0.48±0.18	1.1±0.4	0.79±0.15	0.60±0.06	0.48±0.15
	IC	1.21±0.23	0.81±0.19	0.64±0.21	0.26±0.07	0.11±0.01
Spleen	NC	0.67±0.23	0.23±0.10	0.16±0.03	0.11±0.02	0.014±0.06
	IC	0.3±0.04	0.29±0.08	0.13±0.04	0.047±0.004	0.017±0.006
Lungs	NC	0.29±0.04	0.27±0.07	0.19±0.04	0.11±0.03	0.015±0.003
	IC	—	0.20±0.02	—	0.04±0.01	0.014±0.004
Brain	NC	0.010±0.007	0.013±0.005	0.021±0.009	0.010±0.0015	0.002±0.0017
	IC	0.016±0.007	0.011±0.005	0.012±0.009	0.0048±0.0015	0.0059±0.0017

Results expressed as mean±SD of the percentage of injected doses; $n = 4$–5 mice. NC, native crotamine; IC, irradiated crotamine.
[a]Data statistically significant ($p < 0.05$).

The ^{125}I labeling yield was low, probably due to the avid binding of ^{125}I-crotamine to plastic in consequence of its basic characteristic. Thus, there was a large amount of radioactivity on the plastic tips used during the procedure. The same was observed for the plastic tubes used during purification. This problem was reported by Baker et al. (1993) for ^{125}I-myotoxin *a*. The body distribution profiles of ^{125}I-Nc and ^{125}I-Ic at different time intervals during 24 h are shown in Table 2. The control performed with thyroid gland demonstrated that count rates remained low during the first hours, reaching a maximum peak at 12 h (data not shown).

^{125}I-Nc and ^{125}I-Ic were rapidly absorbed reaching maximum blood concentration 1 h and 1.8 h, respectively, after their administration (Fig. 2). They rapidly decreased during the first 8 h and slowly between 8–24 h, when background levels were reached. These data suggest that native (Nc) and irradiated crotamine (Ic) metabolism and/or elimination occurred within 24 h.

The hepatic and blood profiles of Nc and Ic were slightly different. The Nc was detected in the liver around 30 and 60 min after the injection and its concentration was higher than the blood, while the Ic concentration in the liver was smaller than in the blood during the first 4 h. This can be due to structural alterations on the crotamine molecule induced by gamma radiation. The Nc and Ic appeared to be metabolized in the liver.

The amounts of Nc detected in the kidneys were greater than what was detected in the blood between 0.5 and 4 h, and smaller than what was detected in the liver during 24 h. The amounts of Ic detected in the kidneys were greater than what was detected in blood during the 24 h. Such higher radioactivity levels detected in the kidneys, when using Nc and Ic, suggest that the toxins and/or their possible metabolites have been eliminated by the kidneys, or that the protein has renal clearance. Similar results have been reported by various authors in studies of labeled snake venoms such as *Bungarus multictus* and *Agkistrodon contortrix*, isolated toxins e.g., α and β bungarotoxin, jararafibrase I and scorpion venoms (Lee and Tseng, 1966; Tseng et al., 1968; Wingert et al., 1980; Tanigawa et al., 1994; Ismail and Abd-Elsalam, 1988). This accumulation in the kidney appears to be a common feature for the excretion of foreign proteins.

The comparison between Nc and Ic renal profiles indicate that Ic was eliminated faster than Nc, probably due to structural alterations induced by gamma radiation.

The Nc and Ic were detected in skeletal muscle in the first 6 h, in smaller amounts than blood. Afterwards, the radioactivity in skeletal muscle slowly decreased, and the skeletal muscle profiles were similar to the blood. Both Nc and Ic were detected in lungs, spleen and heart in smaller amounts than what was detected in skeletal muscle, this could indicate the affinity of crotamine for skeletal muscle.

Crotamine induced spasticity and convulsions in mice and rats when injected directly in the brain (Habermann and Cheng-Raude, 1975). Although crotamine shows activity in the central nervous

system when the blood–brain barrier is circumvented, there is little evidence suggesting that it exerts this effect when injected peripherically. In this present paper, insignificant levels of radioactivity, similar to background, was observed in the brain, for all analyzed time intervals for Nc and Ic. This fact suggests that crotamine did not cross the blood–brain barrier and did not present effects to the central nervous system, according to the techniques used in the present study.

In conclusion, the Nc and Ic were rapidly absorbed and they appear to have hepatic metabolism and renal elimination. Both crotamines demonstrated affinity to skeletal muscle and they did not pass the blood–brain barrier. It is possible that the Ic was metabolized and eliminated faster than the Nc, probably by means of structural alterations induced by gamma irradiation, which could possibly explain the reduced toxicity of the irradiated protein.

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