Journal of Natural Toxins, Vol. 6, No. 1, 1997
Printed in USA

COMPARATIVE ANALYSES OF INORGANIC ELEMENTS IN VENOMS FROM THREE SUBSPECIES OF *Crotalus durissus* FROM BRAZIL

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(Received February 1996; Revised manuscript accepted May 1996)

ABSTRACT

Instrumental neutron activation analysis was utilized for determining the concentration of bromine, calcium, cesium, iron, rubidium, antimony, scandium, selenium, zinc, sodium, magnesium, and chlorine in venom samples from subspecies of snakes *Crotalus durissus* (*C. durissus terrificus, C. durissus collilineatus*, and *C. durissus cascavella*), the South American rattlesnakes. No significant difference was observed among them for Sb, Zn, Br, Ca, Cl, and Cs. *C. durissus terrificus* and *C. durissus cascavella* showed no statistical difference for mean concentrations of Sc, Se, and Fe. The concentrations observed for Mg in these subspecies are increased fourfold in relation to the values observed for North American *Crotalus*. The mean Ca and Zn concentrations of Brazilian specimens are similar to values obtained in literature for *Elapidae* venoms. The importance of these elements for the biological activities of venoms is discussed.

INTRODUCTION

Venoms of snakes belonging to the same specie from distinct geographical regions of Brazil, usually classified as subspecies, have presented differences in their venom composition (Barrio, 1960; Schenberg, 1963).

Differences in symptomatology have been described during envenomation by snakes belonging to the same species, especially when they are widely distributed. This discrepancy may be due to differences in venom composition depending on the area of origin of the snakes (Chippaux et al., 1991). From the phylogenetic and

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zoogeographic point of view, *Crotalus* (rattlesnake) snake venom presents the most interesting and important geographical variation (Barrio and Brazil, 1951; Vellard, 1939; Tu, 1982).

The venoms of North American rattlesnakes are mainly hemorrhagic and proteolytic, except for *Crotalus scutulatus* venom that presents a neurotoxic action, whereas South American rattlesnake venoms are mainly neurotoxic (Tu, 1982).

Several inorganic elements have been known for their defined function as enzymatic cofactors (Ingrao et al., 1995). In fact, many enzymes in snake venoms– e.g., phospholipases A_2, phosphodiesterases, 5'-nucleotidases, metalloproteases, and alkaline phosphatases – are dependent on inorganic elements for their activities (Iwanaga and Suzuki, 1979). For example, crotoxin, the main component of *Crotalus durissus* venom, is dependent on calcium for its activity (Breithaupt, 1976; Habermann and Breithaupt, 1978). On the other hand, Zn-dependent metalloproteases play a major role for the hemorrhagic activity of *Crotalidae* snake venoms (Bjarnason and Fox, 1988/89). Thus, the determination of inorganic components in this kind of material becomes of great interest (Freiderich and Tu, 1971). In fact, removing the divalent metal cations (Ca, Zn, and Mg) from snake venoms destroys their hemorrhagic activity (Friederich and Tu, 1971).

There are few papers dealing with the determination of inorganic components in snake venoms. Friederich and Tu (1971) determined the amount of Ca, K, Mg, Na, and Zn by atomic absorption analysis in seventeen different snake species from the *Crotalidae, Viperidae*, and *Elapidae* families. Gitter et al. (1963) applied neutron activation analysis for Cu and Zn determinations in *Naja naja, Vipera palestinae, Echis carinata*, and *Bitis arietans* snake venoms.

Recently, Saiki et al. (1991) have applied the neutron activation method for the determination of Br, Ca, Cl, K, Mg, Na, Rb, Sb, Se, and Zn in pooled venoms from Brazilian snakes of the *Bothrops* and *Crotalus* genera, and have evaluated the accuracy and precision of the method.

The analysis of inorganic elements may produce important data for understanding the role of metals in the pharmacological and biochemical activity of snake venoms, especially in subspecies that proceed from different geographical regions.

This study compares inorganic elements of venom among populations of three subspecies of *Crotalus durissus* (*terrificus, collilineatus*, and *cascavella*) in Brazil.

MATERIALS AND METHODS

Venom Collection

Adult wild snakes (*Crotalus durissus terrificus, Crotalus durissus collilineatus*, and *Crotalus durissus cascavella*), classified according to criteria from Hoge and Romano-Hoge (1978/79), were sent by habitual or sporadic suppliers from different regions in the State of São Paulo, Minas Gerais, and Piauí (Brazil). Once the snakes were received at the Laboratory of Herpetology, they were grouped according to subspecies and region of origin. Venoms from each group of snakes were

extracted manually by squeezing the glands, and collected in Petri dishes. They were dried in a desiccator under vacuum at room temperature. A loss of weight of about 75% is obtained during this drying process.

Element Analysis

Instrumental neutron activation was applied for elemental venom analysis, as described in Saiki et al. (1991). The procedure consisted of irradiating 50 to 150 mg of each pool of venom (weighed in clean plastic bags) in the IEA-R1 nuclear research reactor. During weighing, special care was taken to avoid contamination of samples before the irradiation.

Short irradiations of 5 minutes under a thermal neutron flux of about 2.67×10^{11} $n.cm^{-2}.s^{-1}$ were used for Cl, Mg, and Na determinations, and long irradiations of 16 h under flux of about 10^{13} $n.cm^{-2}.s^{-1}$ were used for Br, Ca, Cs, Fe, Rb, Sb, Sc, Se, and Zn determinations.

After adequate decay times, samples and standards were measured using an EG & G Ortec GMX detector (FWHM of 1.0 KeV at 122 KeV ^{57}Co and of 1.89 KeV at 1332 KeV ^{60}Co) coupled to an EG & G Ortec 918A ADCAM multichannel buffer connected to a 286 AT/IBM microcomputer. Gamma ray spectra were processed using the appropriate computer program, and the concentrations were calculated by the comparative method of neutron activation analysis.

Statistical Analysis

Statistical differences among the three subspecies of *Crotalus durissus* snake venoms were tested by one-way ANOVA and Tukey tests. Transformations were applied when necessary for obtaining homoscedasticity. Kruskall-Wallis ANOVA and Dunn's multiple comparison tests were applied for analyzing Zn, Br, Cl, and Cs. All procedures were carried out by the INSTAT software.

RESULTS

The venoms of three subspecies of *Crotalus durissus* were analyzed for 12 elements by the atomic absorption method. The results are shown in Table 1.

Sodium was the metal found in the highest concentration in the three subspecies studied, reaching milligram levels per gram of venom, together with Mg and Cl. Ca and Zn were in the highest concentrations for the microgram level. The concentrations of Cs, Sb, and Sc were found in nanogram levels.

The elements Sb, Zn, Br, Ca, Cl, and Cs showed no statistical difference in their concentrations. Nevertheless, *C. durissus cascavella* venom exhibited higher mean concentration of Mg and Rb than those found in *C. durissus collilineatus* and *C. durissus terrificus* (Table 2). *C. durissus terrificus* and *C. durissus cascavella* presented no statistical difference for mean concentrations of Sc, Se, and Fe.

DISCUSSION

The concentration of inorganic elements produced by snake venom glands surely plays a role for the development of venom activities by metalloproteases, since enzymes depend on metal ions as cofactors.

Table 1. Concentration of inorganic elements in venoms from three subspecies of *Crotalus durissus* snakes.

Elements	*Snake venoms*		
	C .d. terrificus	*C. d. collilineatus*	*C. d. cascavella*
Na (mg/g)	21.93±3.50 (18.34-30.65) n=15	18.88±2.63 (13.03-23.17) n=19	13.65±5.02 (10.06-25.30) n=8
Mg (μg/g)	4051.7±673.7 (2889-5175) n=11	6375.6±1224.8 (3644-7848) n=15	8997.4±3171.5 (3658-12350) n=7
Rb (μg/g)	11.87±10.01 (4.71-45.22) n=14	9.10±3.22 (4.54-16.87) n=18	4.71±2.96 (2.60-10.83) n=8
Sb (ng/g)	24.61±20.20 (4.70-56.30) n=8	13.50±8.69 (1.67-37.30) n=13	18.99±7.30 (8.55-26.30) n=6
Sc (ng/g)	3.67±3.68 (1.21-11.41) n=12	1.32±0.75 (0.58-3.50) n=20	2.86±2.26 (1.26-8.00) n=8
Se (μg/g)	4.63±1.18 (2.52-6.06) n=16	5.76±1.11 (3.06-6.93) n=17	3.97±1.71 (1.79-6.80) n=10
Zn (μg/g)	104.66±50.42 (60.85-207.50) n=12	78.62±17.53 (48.11-101.60) n=18	55.97±43.65 (12.03-142.00) n=8
Br (μg/g)	9.20±7.16 (1.58-23.80) n=15	4.74±1.01 (2.95-6.71) n=29	7.00±7.99 (1.56-19.30) n=7
Ca (μg/g)	440.22±162.56 (290.0-750.0) n=9	484.71±194.76 (272.0-986.0) n=21	402.78±89.58 (234.0-479.0) n=9
Cl (μg/g)	2805.45±3229.60 (438.0-8598.0) n=11	1397.47±202.39 (994.0-1709.0) n=19	1775.43±1498.90 (565.0-4123.0) n=7
Cs (ng/g)	33.48±17.49 (16.96-68.10) n=12	82.09±100.82 (3.48-299.0) n=13	40.90±19.00 (13.70-59.80) n=9
Fe (μg/g)	16.04±15.36 (4.19-43.94) n=10	5.24±2.00 (2.89-9.41) n=18	24.68±21.50 (4.25-55.12) n=7

Data are expressed as mean ± standard deviation, range (in parentheses) and number of determinations.

Table 2. Statistical differences among venoms from three subspecies of *Crotalus durissus* snakes.

Elements	*Comparisons*		
	C. d. cascavella vs. C. d. collilineatus	*C. d. cascavella vs. C. d. terrificus*	*C. d. terrificus vs. C. d. collilineatus*
Na	p<0.01	p<0.001	p<0.05
Mg	p>0.05	p<0.001	p<0.001
Rb	p<0.01	p<0.001	p>0.05
Sb	p>0.05	p>0.05	p>0.05
Sc	p<0.05	p>0.05	p<0.01
Se	p<0.01	p>0.05	p<0.05
Zn	p>0.05	p>0.05	p>0.05
Br	p>0.05	p>0.05	p>0.05
Ca	p>0.05	p>0.05	p>0.05
Cl	p>0.05	p>0.05	p>0.05
Cs	p>0.05	p>0.05	p>0.05
Fe	p<0.01	p>0.05	p<0.05

p>0.05 was considered non-significant.

Among the biological parameters studied by Friederich and Tu (1971), the lethality was not affected by the remotion of metals. A significant decrease in the hemorrhagic activity was observed when Ca, Zn, and Mg were removed by EDTA. However, the proteolytic activity can be restored by the addition of Mg or Zn.

The values in literature for inorganic elements within *Crotalus* genus show an intense variation throughout America, even for specimens coming from nearby regions (Friederich and Tu, 1971). In fact, this variation may be related to the huge variation of activities showed by this genus: hemorrhagic and proteolytic in North America, and neurotoxic in South America, resembling *Elapidae* venoms.

Among the twelve elements studied, six (Sb, Ca, Zn, Br, Cl, and Cs) presented no statistical difference for their means in the subspecies of *Crotalus durissus* studied in this paper.

Friederich and Tu (1971) reported a systematic investigation of metal contents in venoms of various snakes with wide geographical distribution. They noted that nondialyzed venoms from *Crotalidae* (rattlesnakes and copperheads) contained more calcium and zinc than *Elapidae* venoms. In the present study, determined values of Ca ranged from 234 to 986 μg/g and Zn values ranged from 12 to 207.5 μg/g of venom. However, these data are lower than those reported by Friederich and Tu (1971) for other nondialyzed venoms – *Elapidae*: 1000-1620 μg Ca and 196-1600 μg Zn/g; *Crotalidae*: 150-4930 μg Ca and 680-2010 μg Zn/g; *Viperidae*: 1987-2900 μg Ca and 690-1800 μg Zn/g – and by Rodriquez et al. (1974) for Venezuelan *C. durissus cumanensis* (4500 μg Ca/g and 780 μg Zn/g). Interestingly,

the values shown by the Brazilian subspecies of *C. durissus* for Ca are quite similar to those observed for *C. horridus atricaudatus* (150 μg/g) by Friederich and Tu (1971).

According to some authors, the metal content of venoms may vary according to the diet that snakes are submitted (Elliott, 1978; Kumar and Elliott, 1973). In fact, different zinc contents may be observed regarding whether specimens are maintained in captivity or not (Kumar and Elliott, 1973).

In regard to Mg and Na, *Crotalus durissus terrificus* was the most different among the three subspecies studied. The lowest values of Mg found in *C. d. terrificus* (2889-5175 μg/g) were at least four times higher than those obtained for other *Crotalus* species (Friederich and Tu, 1971; Rodriguez et al., 1974). In fact, Mg is usually present in lesser amounts in other venoms, varying from 107 μg/g in the venom of *Crotalus adamanteus* to 1470 μg/g in the venom of *Crotalus durissus* (Central America) (Friederich and Tu, 1971; Rodriguez et al., 1974). At the moment, the relationship between the high Mg concentration of venoms from the three Brazilian *Crotalus durissus* subspecies and their biological activities has not been established.

The Na values obtained for Brazilian *Crotalus durissus* subspecies were within the range obtained for *Crotalus* genus (Friederich and Tu, 1971; Rodriguez et al., 1974). The difference for mean Na concentration among the three subspecies may be explained by variation of food intake due to their different geographical distribution.

In regard to Sc, Se, and Fe, *C. durissus collilineatus* seemed to be the most different of the three subspecies: the highest values for Se, and the lowest values for Sc and Fe (Table 1). In fact, the methodology utilized herein seemed more sensitive for the determination of Fe and Se than atomic absorption (Friederich and Tu, 1971). The reason for the high concentration of Fe observed in *C. durissus cumanensis* (490 μg/g) by Rodriguez et al. (1974) is a matter of speculation.

The taxonomic classification of subspecies of *Crotalus durissus* is based exclusively on their morphological external aspects and geographical distribution (Hoge and Romano-Hoge, 1978/79). This study was carried out in an attempt to observe if these subspecies presented some difference in the composition of inorganic elements in their venoms. In fact, the inorganic elements could not differentiate these subspecies, since six of these elements presented no statistical difference. The other elements that presented statistical significance could not differentiate substantially these subspecies.

C. durissus terrificus venom does not induce the characteristic signs of *Crotalidae* envenomation, i.e., edema and hemorrhage. Systemic envenomation by this species resembles more closely those of elapids, causing little swelling and only occasional mild pain (Hendon and Bieber, 1982; Tu, 1982; Arnold, 1982). Clinical pictures for *C. durissus collilineatus* and *C. durissus cascavella* have not been described in literature. Sanchez et al. (1992) observed that the edematogenic activity of *Crotalus durissus* subspecies was lower than in other *Crotalinae*. In opposition to most of the North American *Crotalus* species, the South

American *Crotalus durissus* has no caseinolytic or hemorrhagic activities (Sanchez et al., 1992).

When the concentrations of Ca and Zn from Brazilian subspecies of *C. durissus* are compared to the results obtained from North American *Crotalidae*, it may be observed that the South American *Crotalus durissus* shows lower values. It is a matter of speculation if it can be related to the neurotoxic activity found in both venoms. Further investigations should be carried out to know if a relationship between the inorganic composition of the venoms and their biological activity exists.

This fact allows us to suggest that the hemorrhagic and proteolytic activities demonstrated by North and Central America (Ownby, 1982; Sanchez et al., 1992; Glenn and Straight, 1982; Lomonte and Gutierrez, 1983), as well as the neurotoxic activity observed in South American rattlesnakes (Sanchez et al., 1992), may be partially controlled by the concentration of inorganic components on the venom proteins responsible for these activities. The assay of inorganic elements in *C. scutulatus* venom might contribute to solve this question.

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