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Individual venom variability in the South American rattlesnake *Crotalus durissus cumanensis*

Irma Aguilar[a], Belsy Guerrero[b], Ana Maria Salazar[b], Maria E. Girón[a], John C. Pérez[c], Elda E. Sánchez[c], Alexis Rodríguez-Acosta[a,*]

[a]*Sección de Inmunoquímica, Instituto de Medicina Tropical de la Universidad Central de Venezuela, Apartado 47423, Caracas 1041, Venezuela*

[b]*Laboratorio de Fisiopatología, Centro de Medicina Experimental, Instituto Venezolano de Investigaciones Científicas (IVIC), Apartado 21827, Caracas 1020A, Venezuela*

[c]*Natural Toxins Research Center (NTRC), Texas A&M University-Kingsville, MSC 158, Kingsville, TX 78363, USA*

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Abstract

Crotalus durissus cumanensis snake venoms from different Venezuelan regions, showed biochemical and hemostatic variations. Fibrino(geno)lytic, hemorrhagic and procoagulant activities and gel-filtration chromatography and SDS-PAGE profiles were analyzed. Differences were observed in fibrinolytic activity: kallikrein-like amidolytic activity was highest in venoms of Santa Teresa, and Margarita. Lagunetica and Carrizales venoms showed the maximum fibrin lysis. The highest hemorrhagic activity was seen in Lagunetica venom. Margarita had the lowest LD_{50} of 0.18. Lagunetica, Carrizales and Anzoátegui induced a rapid degradation of fibrinogen α chains and slower degradation on β chains, which could possibly due to a higher content of α fibrinogenases in these venoms. This fibrinogenolytic activity is decreased by metalloprotease inhibitors. All venoms, except Carrizales, presented thrombin-like activity. Anzoátegui, Carrizales and Lagunetica, in which fibrinolytic activity was present, showed the largest concentration of high molecular mass components. These results represent a new finding, not previously described, of fibrinolytic activity in South American *C. durissus* venoms. Santa Teresa and Margarita had fibrinolytic activity, and lack of hemorrhagic activity, representing an important finding in Venezuelan venoms since the description of a fibrinolytic molecule without hemorrhagic activity can have valuable potential in thrombolytic therapy.

Keywords: Coagulation; *Crotalus durissus cumanensis*; Fibrino(geno)lytic; Hemostasis; Hemorrhage; Rattlesnakes

1. Introduction

Rattlesnakes, belonging to the genus *Crotalus* (Viperidae family) are geographically distributed from Canada to northern Argentina. *Crotalus durissus* has the broadest range of geographical distribution, in which 14 subspecies have been described (Campbell and Lamar, 1989). This ample

*Corresponding author. Tel.: +58 212 6053632; fax: +58 212 6053550.
E-mail address: rodriguezacosta1946@yahoo.es (A. Rodríguez-Acosta).

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geographical distribution has generated the existence of intraspecific variability in the venom composition and its effects with consequent clinical and therapeutic implications (Chippaux et al., 1991; Francischetti et al., 2000; Saravia et al., 2002; Dos-Santos et al., 2005).

Venoms from similar species of diverse geographical areas could differ in their constituents and consequently their toxicity (Salazar et al., 2006). Several authors (Campbell and Lamar, 1989; Salazar et al., 2006) describe that venom individualities are not consistent indicators of taxonomic associations due to intraspecific (individual) differences of snake venoms. One of the factors that can influence venom toxicity and cause variable results is the environmental conditions. It would be possible that geographical variation in venom composition reflects natural selection for feeding on local prey (Daltry et al., 1996; Pifano and Rodriguez-Acosta, 1996). However, a comparative analysis of enzymatic and other toxic activities of venoms of the same species of *C. durissus cumanensis* found in diverse Venezuelan geographical areas is still absent.

The Venezuelan rattlesnake (*C. d. cumanensis*) is broadly extended across the country. In specific geographical areas, this species produces obvious effects on a victim's hemostatic system (Yoshida-Kanashiro et al., 2003; Aguilar et al., 2006).

Hemorrhagic and fibrinolytic activities play an important role in Viperidae snake envenomation, promoting coagulopathies and local and systemic bleeding (Otero et al., 1992; Gutierrez et al., 1995; Saravia et al., 2002). It is remarkable to observe that the hemorrhagic activity in venom of the genus *Crotalus* (widely described in the American continent) intensifies from south to the north, and is highly present in North American *Crotalus*, and yet, almost non-existent in the majority of the South American *Crotalus* species and subspecies (Rodriguez-Acosta et al., 1998). Since death is not a comparatively common result of envenomation, emphasis has been placed on research involving the significant systemic effects caused by envenomation such as hemorrhagic diathesis and disseminated intravascular coagulation.

The present work seeks to evaluate in *C. d. cumanensis* crude venoms from different geographical areas of Venezuela, the possible differences among components affecting the hemostatic system, using plasma, fibrinogen and fibrin as natural substrates and synthetic substrates, with the purpose of determining their possible mechanisms of action on hemostasis. This would facilitate a better understanding of the clinical picture and it could increase the antivenom specificity and reactivity, assuring the neutralization of the relevant pathological alterations.

2. Materials and methods

2.1. Reagents

Chromogenic substrates, and human fibrinogen (10% w/w of plasminogen as contaminant) were purchased from Chromogenix AB (Milano, Italy). Molecular mass standards for SDS-PAGE were from Bio-Rad Laboratories Ltd. (California, USA). Purified substrates Xa, bovine alpha thrombin, single chain t-PA (sctPA), two chains u-PA (tcu-PA) and plasmin (used for standard controls), were acquired from American Diagnostica Inc. (Greenwich, CT, USA). Aprotinin, phenylmethylsulfonyl fluoride (PMSF), 1-10 phenantroline, benzamidine/HCl, ethylene glycol-bis-N,N,N′,N′-tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA) and other reagents used in this study were from Sigma Chemical Co (St. Louis, MO, USA).

2.2. Animals

Albino Swiss NIH strain male mice between 18 and 22 g were obtained from the National Institute of Hygiene "Rafael Rangel", Caracas, Venezuela. The investigation complies with the bioethical norms taken from the guide "Principles of laboratory animal care" (Anonymous, 1985).

2.3. Snakes

Crotalus d. cumanensis venoms from adult snakes were obtained from Lagunetica, Santa Teresa, Carrizales, and Guarenas (Miranda state); Anzoátegui (Anzoátegui state); Margarita (Nueva Esparta state); Maracay (Aragua state) Venezuelan geographical locations (Fig. 1). All venoms were pooled samples (from six individual adult). Venom pools were filtered through a 0.45-μm membrane, lyophilized, divided into 30 mg samples and stored at −80 °C.

2.4. Protein concentration determination

The protein concentration was determined by the method of Lowry et al. (1951).

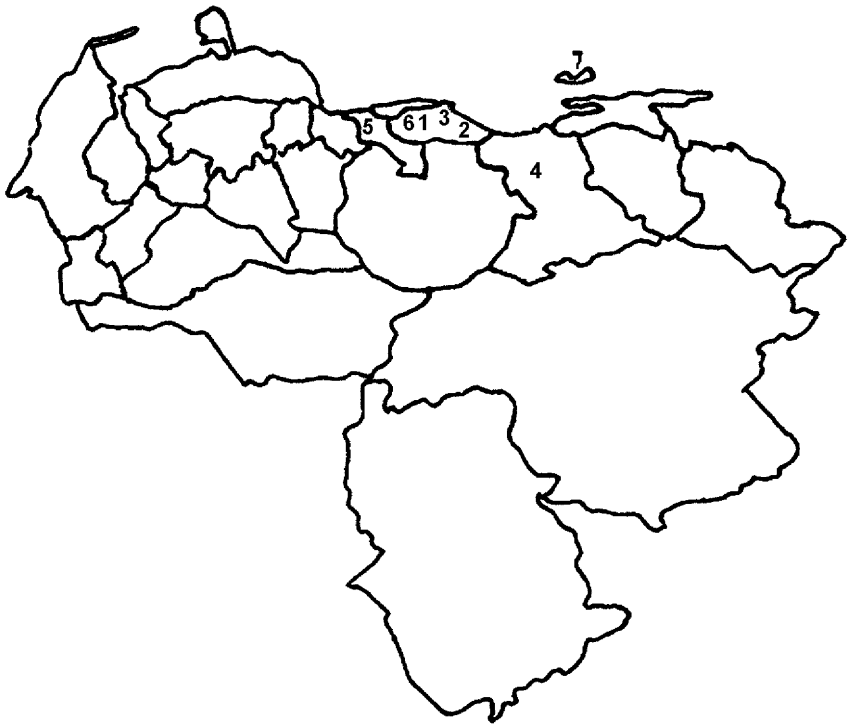

Fig. 1. Distribution of *C. d. cumanensis* specimens in this study. Lagunetica (1), Santa Teresa (2), Guarenas (3) and Carrizales (6) venoms (Miranda state); Aragua (5) venom (Aragua state); Anzoátegui (4) venom (Anzoátegui state) and Margarita (7) venom (Nueva Esparta state).

2.5. Determination of MHD

To determine the minimal hemorrhagic dose (MHD) for the crude venoms, the Omori-Satoh et al. (1972) method was used. A series of eight dilutions were made for each snake venom, 0.1 mL of each dilution was intracutaneously injected into the depilated backs of mice. The mouse was sacrificed and the skin removed after 24 h. The hemorrhagic diameter on the skin was measured and the MHD determined. The MHD is defined as the amount of venom protein that causes a 10 mm hemorrhagic spot.

2.6. Determination of lethality

Venom lethality was determined in mice by intraperitoneal injections of the sample, and the LD_{50} value calculated according to the method of Spearman-Karber (1978).

2.7. Amidolytic activity

Amidolytic activity of crude snake venom was measured by a micromethod standardized in our laboratory (Guerrero and Arocha-Piñango, 1992). Briefly, in 96 wells polystyrene plates a mixture of 80 μL of the recommended buffer for each substrate, 10 μL of the venom sample (0.1 mg/mL, 0.5 mg/mL or 1 mg/mL) and 10 μL of substrate (final concentrations of 0.6 mM S-2238, 1.2 mM S-2288, 0.16 mM S-2586, 0.8 mM S-2222, 0.80 mM S-2251 or 0.16 mM S-2444) were placed in each well. Bovine thrombin, human factor Xa, sct-PA, tcu-PA, and plasmin were used as positive controls. After incubation at 37 °C for 15 or 30 min, the absorbance at 405 nm was measured. One unit of amidolytic activity was expressed as ΔA 405 nm/min. Specific activity was calculated as units/mg of protein.

2.8. Fibrinolytic activity

Fibrinolytic activity of crude snake venom was studied by the fibrin plate method as described by Marsh and Arocha-Piñango (1972). Briefly, fibrin plates were prepared using 3-cm diameter Petri dishes: 1.5 mL of a 0.1% plasminogen-rich fibrinogen in imidazol saline buffer, pH 7.4 was clotted by

adding 75 μL bovine thrombin (10 U/mL, in 0.025 M $CaCl_2$). The mixture was incubated at room temperature for 30 min. Then, a 10 μL sample was applied over the fibrin film, and after 24 h incubation at 37 °C the diameter of the lysed areas was measured. Fibrinolytic activity was expressed as the diameter of the lysed area per microgram of protein ($mm^2/\mu g$). Human plasmin, t-PA (sct-PA) and u-PA (tcu-PA) were used as positive controls.

2.9. Fibrinogenolytic activity

Effect of *C. d. cumanensis* crude venom on fibrinogen chains was evaluated using a human fibrinogen solution in 0.05 M imidazole-0.15 M NaCl, pH 7.4 buffer and venom solutions that were previously treated with 10 mM PMSF in order to inhibit the thrombin-like activity. Briefly, fibrinogen was incubated with venom at 1 μg venom/100 μg fibrinogen ratio, at different times at 37 °C in the presence or absence of proteases inhibitors. The degradation of the fibrinogen chains was visualized by SDS-PAGE on a 10% gel under reduced conditions using Tris-Tricine-system (Schägger and von Jagow, 1987). A serine protease inhibitor pool was used at 50 μg/mL SBTI, 10 mM PMSF, 10 mM benzamidine, and 100 U/mL aprotinine (final concentration). Metalloprotease inhibitors were used as a mixture of 10 mM EDTA-Na; 10 mM EGTA-Na and 10 mM 1,10 phenantroline (final concentration).

2.10. Coagulant activity on plasma and fibrinogen

The thrombin-like coagulant activity in venoms was determined by the method adapted from Austen and Rhymes (1975). Briefly, 0.1 mL of 0.05 M Tris-HCl buffer, pH 7.4 (coagulation buffer) plus 0.1 mL thrombin solution (0.5–15 IU/mL) or 0.1 mL venom sample (diluted in coagulation buffer) was incubated in a borosilicate tube at 37 °C. Then 0.1 mL of fresh citrate human plasma or 0.3% human fibrinogen solution in coagulation buffer was added. The solution was thoroughly mixed and clotting time recorded. Tests were performed four times and the mean clotting time calculated. The results were expressed in thrombin-like units by plotting the clotting times against a calibration curve prepared with a standard thrombin (National Institute for Biological Standards and Control, London, England).

2.11. Chromatographic venom profiles

Samples from *C. d. cumanensis* venoms were run molecular exclusion chromatography on Superose 12 HR10/30 column equilibrated with 50 mM ammonium acetate buffer pH 6.8, in order to compare their chromatographic profiles. Venom samples (5 mg/100 μL) were dissolved in equilibrium buffer and injected into the column. The elution was carried out with the same solution at 0.4 mL/min flow rate and monitored at 280 nm.

2.12. SDS-PAGE analysis

Polyacrylamide gel electrophoresis of venom samples were carried out following Laemmli (1970) method using a Mini-Protean II system (Bio-Rad Laboratories, Hercules, CA). Venom samples were diluted in 0.063 M Tris–HCl buffer, pH 6.8, containing 2% SDS, 5% glycerol and 0.001% bromophenol blue and then boiled for 4 min before electrophoresis at 100 V (constant). After electrophoresis, the gels were stained with 0.1% brilliant blue Coomassie R250 in acetic acid:ethanol:water (5:25:70, v/v) and then distained in the same solution.

2.13. Statistical analysis

The activities were statistically described according to their mean and standard deviation. Variance analysis was examined by a one-way ANOVA analysis followed by Student–Newman–Keuls multiple comparisons test. Results represent mean ± SD ($n = 4$). p values of 0.01 or less were considered statistically significant (GraftPad Prism, USA).

3. Results

3.1. Hemorrhagic activity

The MHD present in Lagunetica, Anzoátegui and Carrizales venoms were of 4.1, 16.2 and 14.3 μg/mouse, respectively. In contrast, Santa Teresa, Guarenas, Aragua, and Margarita venoms did not present hemorrhagic activity (Table 1).

3.2. Lethal activity

The LD_{50} calculated for Lagunetica, Santa Teresa, Guarenas, Anzoátegui, Aragua, Carrizales and Margarita venoms were 0.86, 0.43, 0.66, 0.60,

0.66, 0.86 and 0.18 mg/Kg, respectively (Table 1). Margarita venom significantly showed the lowest LD_{50} ($p<0.001$).

3.3. Fibrinolytic activity

The fibrinolytic activity was evaluated by a fibrin plate method and the amidolytic method with S-2251, S-2302, S-2444 and S-2288 substrates, which determined the plasmin-like, kallikrein-like, urokinase-like and t-PA-like activities, respectively. The results in Table 2 demonstrate that the highlight amidolytic activity was kallikrein like, followed by low t-PA-like and plasmin-like activities. When a comparative analysis of the seven venoms from different regions was carried out, it was observed that kallikrein-like activity was significantly higher ($p< 0.01$) in Margarita and Santa Teresa than Lagunetica and Carrizales venoms.

The results of fibrin plate showed that Lagunetica and Carrizales venoms significantly ($p<0.001$) presented the highest fibrinolytic activity. Additionally, Carrizales was more active than Lagunetica ($p<0.05$). Fig. 2 shows fibrinolytic plate results.

Table 1
Comparison of fibrinolytic activity, MHD and LD_{50} of *C. d. cumanensis* venoms

Crotalus venoms	Fibrinolytic activity[a], 10 μg sample	MHD[b] (μg/mouse)	LD_{50}[c] (mg/Kg)
Lagunetica	51.3±4.0	4.1	0.86
Santa Teresa	6.4±2.5	Neg	0.43
Guarenas	14.7±2.3	Neg	0.66
Anzoátegui	25.0±1.0	16.2	0.60
Aragua	32.4±5.2	Neg	0.66
Carrizales	49.2±6.3	14.3	0.86
Margarita	3.4±1.1	Neg	0.18

Data are expressed as mean±SD ($n=4$).
[a]Fibrin plate method: 10 μg sample. Fibrinolytic activity was evaluated on plasminogen rich-fibrin plates using Marsh and Arocha-Piñango (1972) method.
[b]MHD: Minimum hemorrhagic dose, determined by Omori-Satoh et al. (1972).
[c]LD_{50}: Lethal doses 50, determined by Spearman-Karber (1978) method.

3.4. Fibrinogenolytic activity

Fibrinogen degradation studies carried out with Lagunetica, Anzoátegui (the lowest thrombin-like activity) or Carrizales venoms (no coagulant activity), under reduced conditions showed that α and β chains were degraded in a time-dependent action by all venoms. In the SDS-PAGE obtained from fibrinogen treated with Carrizales venom it was observed that α chain degradation started at 0.5 min, with a maximum at 5 min (Fig. 3A, lane 4) and degradation of β chains was from 30 to 240 min where at 240 min full degradation was observed; γ chains were unaffected. This fibrinogenolytic activity was completely inhibited by metalloproteases inhibitors (Fig. 3B). Serine proteases inhibitors did not modify the degradation pattern (data not shown).

Table 2
Fibrinolytic activity of *C.d. cumanensis* venoms

Crotalus venoms	Amidolytic method[a] (UA/min/μg)				Fibrin plate ($mm^2/\mu g^{b,c}$)
	S-2251 (Plasmin)	S-2302 (Kallikrein)	S-2444 (Urokinase)	S-2288 (t-PA)	
Lagunetica	32.7±2.0	306.7±7.8	15.3±5.2	42.1±6.3	3.7±0.2 **
Santa Teresa	59.4±1.2	510.0±6.0*	18.1±5.5	69.3±6.6	1.2±0.1
Guarenas	50.9±1.4	442.0±5.6	17.9±4.7	50.4±8.2	2.2±0.1
Anzoátegui	42.9±1.0	390.7±6.0	16.1±4.5	44.5±6.8	2.5±0.1
Aragua	53.1±1.2	460.6±7.6	19.6±7.4	56.0±6.6	2.6±0.2
Carrizales	24.1±0.5	192.4±5.6	14.2±7.0	27.3±7.4	4.3±0.2**·***
Margarita	66.5±4.2	524.0±7.2*	20.1±8.8	76.5±7.2	1.4±0.1

Data are expressed as mean±SD ($n=4$).
*$p<0.01$ in comparison with Lagunetica and Carrizales venoms.
**$p< 0.01$ in comparison with other venoms.
***$p<0.05$ in comparison with Lagunetica venom.
[a]Amidolytic activity was expressed as ΔA 405 nm/min/mg protein.
[b]Fibrinolytic activity was evaluated on plasminogen rich-fibrin plates using Marsh and Arocha-Piñango (1972) method.
[c]This is the mean value of 9 experiments (3 doses $n=4$).

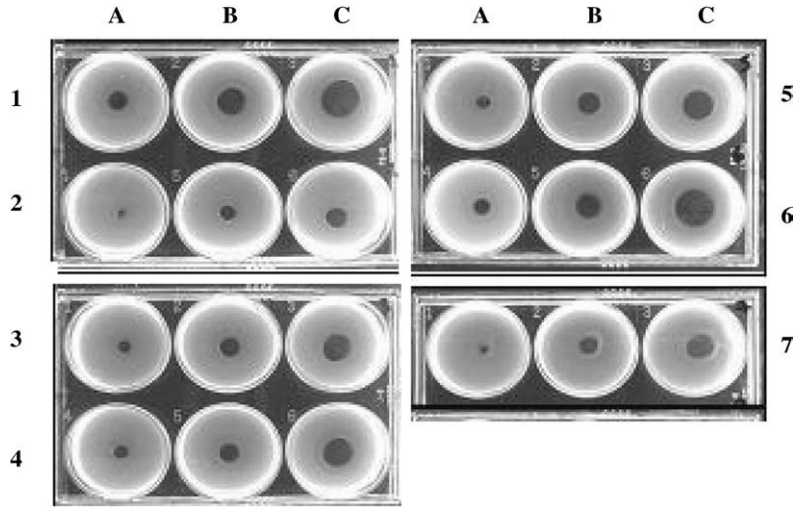

Fig. 2. Fibrinolytic activity of *C.d. cumanensis* venoms: 10 (A), 25 (B) and 50 (C) μg, in plasminogen-rich fibrin plates. Venoms: (1) Lagunetica; (2) Santa Teresa; (3) Guarenas; (4) Anzoátegui; (5) Aragua; (6) Carrizales; and (7) Margarita.

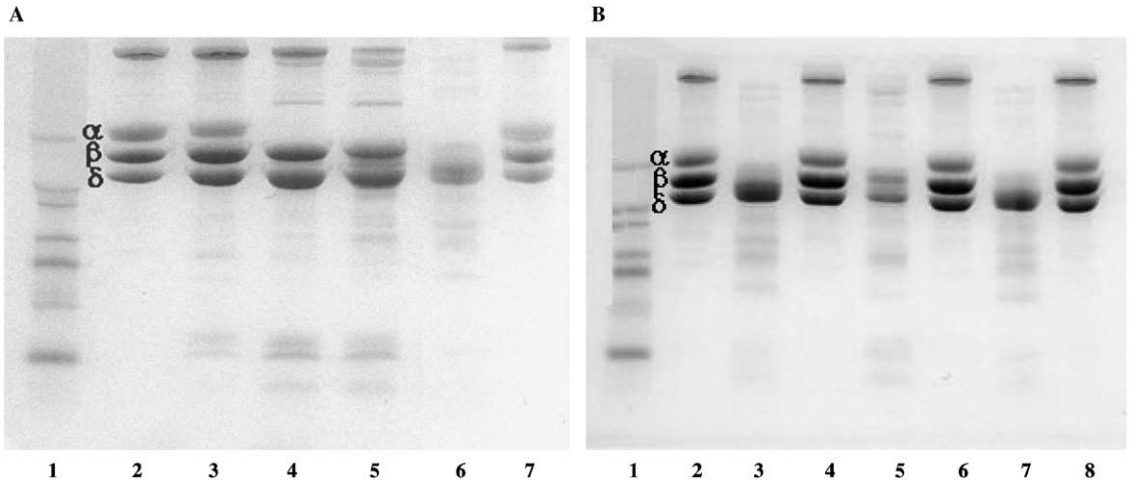

Fig. 3. Fibrinogenolytic activity of *C.d. cumanensis* venoms. Fibrinogen (Fg) treated with Carrizales venom (CV) at 1 μg venom/100 μg Fg ratio at 37 °C. Samples (50 μg) were electrophoresed under reduced conditions in the presence of SDS on 10% gel and stained with Coomassie Blue. Fibrinogen consists of three polypeptide chains Aα, Bβ and γ. A. Fibrinogen after of treated with Carrizales venom during different times of incubation. Lanes: (1) LMW markers; (2) Fg + CV to 0 min; (3) Fg + CV to 0.5 min; (4) Fg + CV to 5 min; (5) Fg + CV to 30 min; (6) Fg + CV to 240 min; (7) Control Fg. B. Fibrinogen after treating with Lagunetica (LV), Anzoátegui (Anz V) and Carrizales (Carr V) venoms at 1 μg venom/100 μg Fg ratio during 240 min at 37 °C, in presence or absence of metalloprotease inhibitors (MPI). Lanes: (1) LMW markers; (2) Fg Control; (3) Fg + LV; (4) Fg + (LV + MPI); (5) Fg + AnzV; (6) Fg + (AnzV + MPI); (7) Fg + CarrV; and (8) Fg + (CarrV + MPI).

3.5. Procoagulant activity

An amidolytic method using the substrates S-2222 and S-2238, specific to detect factor Xa and thrombin, respectively, was employed to determine the procoagulant activity. This activity was also assayed using the clotting method with citrated human plasma or human fibrinogen as substrate. The results in Table 3 show a thrombin-like activity under S-2238, fibrinogen and plasma. In Santa Teresa, Guarenas and Aragua venoms the procoagulant activity was higher with fibrinogen in comparison to the plasma. Furthermore, not any of the venoms presented factor Xa-like activity (data not shown).

When a comparative analysis of venoms was carried out, it was observed that the procoagulant

activity (plasma or fibrinogen) in Santa Teresa, Guarenas and Aragua had the highest activity ($p<0.01$). Thrombin-like (S-2238) activity was significantly higher ($p<0.05$) in Santa Teresa, Aragua and Margarita venoms compared with Lagunetica, Anzoátegui and Carrizales ($p<0.001$).

Table 3
Procoagulant activity of *C.d. cumanensis* venoms

Crotalus venoms	Coagulant method IU thrombin/mg[a]		
	S-2238 (Thrombin)	Plasma	Purified fibrinogen
Lagunetica	58.3±5.2	3.4±0.1	<1
Santa Teresa	107.5±5.6**	24.0±0.1*	31.3±0.2*
Guarenas	95.8±5.2	23.8±0.2*	30.2±0.2*
Anzoátegui	68.5±5.4	3.8±0.2	3.4 ±0.2
Aragua	105.5±5.4**	18.6±0.2*	30.6±0.2*
Carrizales	31.4±5.2	Incoagulable	Incoagulable
Margarita	102.7±5.6**	12.6±0.4	16.2±0.3

Data are expressed as mean±SD ($n=4$).
*$p<0.01$ compared with other venoms.
**$p<0.001$ compared with Lagunetica, Anzoátegui and Carrizales venoms.
[a]Coagulant activity was determined by Austen and Rhymes (1975). The results are expressed in thrombin-like units by plotting the clotting times against a calibration curve prepared with a standard thrombin (National Institute for Biological Standards and Control, London, England).

3.6. Venom chromatographic profiles

The molecular exclusion column Superose 12 HR10/30 chromatographic profiles from the seven studied venoms are shown in Fig. 4. Seven main peaks with elution times of 23.28, 26.08, 27.12, 32.12, 33.10, 37.20 and 40.48 min in all venoms were detected, which were classified according to their molecular mass in: very high (VH), high1 (H1), high2 (H2), intermediate1 (I1), intermediate2 (I2), medium (M), and low (L), respectively.

Quantitative variations among the venoms were evidenced. The VH peaks showed the highest protein concentration in Lagunetica, Carrizales and Anzoátegui venoms. Lagunetica and Carrizales showed a lower concentration of H1 and H2 peaks. Anzoátegui presented a highest concentration of peak H1 and H2 (seen in a single peak). Santa Teresa, Guarenas and Anzoátegui showed an I2 peak that did not appear in the other venoms. Santa Teresa also shows the peak H2 in high concentration. Guarenas and Santa Teresa showed the VH peak in a lower concentration. Aragua and

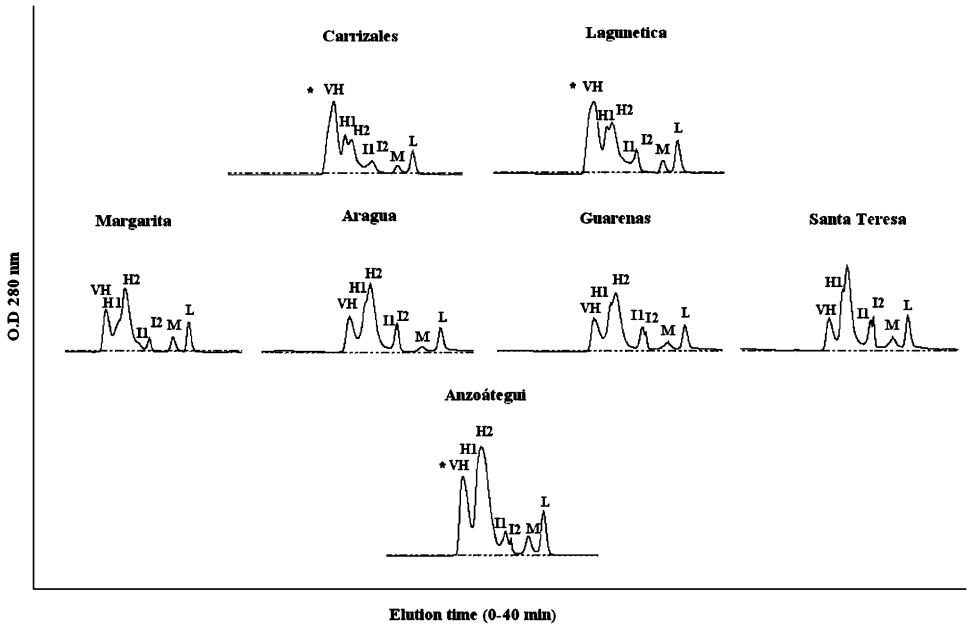

Fig. 4. Size exclusion chromatographic profiles of *C.d. cumanensis* venoms. Ten milligrams of each venom were dissolved in 50 mM ammonium acetate buffer, pH 6.8 and dispensed in a Superose 12 HR 10/30, previously equilibrated with 50 mM ammonium acetate buffer, pH 6.8. The venom was run for 60 min and proteins were detected at 280 nm. The peaks were classified according to their molecular mass; VH: very high; H1: high1; H2: high2; I1: intermediate1; I2: intermediate2; M: medium; L: low. * Peaks with fibrinolytic activity.

Margarita were similar due to the presence of intermediate protein concentrations in most of the peaks, except in M peak, which was slightly lower in Aragua than Margarita and I1 peak that was lower in Margarita than Aragua. Carrizales, Lagunetica and Anzoátegui showed fibrinolytic activity in their highest molecular weight peaks (Fig. 4).

3.7. Electrophoretic analysis

The SDS-PAGE patterns under reduced conditions pointed up some differences among the seven studied venoms (Fig. 5). Lagunetica, Anzoátegui and Carrizales venoms showed comparable profiles with similar intensity of bands. Santa Teresa, Guarenas and Aragua venoms did not show the 24 kDa band. Margarita venom showed different band intensities, presenting a very weak intensity in the 24 kDa band. Carrizales did not show the 116 kDa band.

4. Discussion

Some reports of *Crotalus* envenomation in patients from Venezuela (Miranda state), describe increase of clotting time, partial thromboplastin time (PTT), prothrombin time (PT), fibrinogen consumption, platelets and small hemorrhages in the area of the bite (Yoshida-Kanashiro et al., 2003). Experiments using some fractions of *Crotalus* venom have shown that they were able to cause

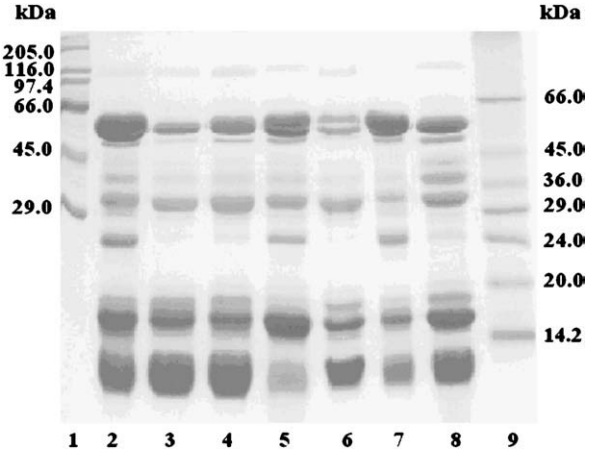

Fig. 5. SDS-PAGE (12%) of *C. d. cumanensis* venoms: 50 μg venom was carried out under reducing conditions. Gels were stained with brilliant blue Coomassie. Lines: (1) High molecular weight markers (2) Lagunetica; (3) Santa Teresa; (4) Guarenas; (5) Anzoátegui; (6) Aragua; (7) Carrizales; (8) Margarita; and (9) Low molecular weight markers.

hemorrhage (Rodriguez-Acosta et al., 1998; Aguilar et al., 2001). Amaral et al. (1988) reported that spontaneous bleeding has rarely been observed in *C. durissus terrificus* envenomed patients, and some patients did not present detectable fibrinogen and thrombocytopenia after the accident. According to Kamiguti and Sano-Martins (1995), South American rattlesnake venoms produce blood incoagulability due to fibrinogen consumption.

Most recent attention has been focused in snake venom fibrinolytic metalloproteases because of their clinical potential in treating thrombotic vascular syndrome (Mori et al., 1987; Willis et al., 1989; Markland, 1998; Matsui et al., 2000; Swenson and Markland, 2005). At the present time, fibrinolytic activity in South American *Crotalus* venom has not been reported. For this reason, one of the main aims of this paper was to evaluate this activity in *C. d. cumanensis* venom from different Venezuelan locations. Our findings demonstrated that all *C. d. cumanensis* venoms presented a fibrinolytic activity by amidolytic or fibrin plate methods. The results with chromogenic substrates showed in all venoms, a high kallikrein-like activity and a low t-PA-like activity. On fibrin plasminogen rich plates, the venoms were also active (Fig. 2). In addition, among venoms of different geographic areas appreciable variations were observed, thus the kallikrein-like amidolytic activity was more elevated in Santa Teresa and Margarita venoms; in contrast, greater fibrin lysis was observed in Lagunetica and Carrizales venoms. The high kallikrein-like amidolytic activity could be producing an effect on the contact system, activating factor XII, prekallikrein or kinin system. It has been demonstrated *in vitro* that the activity of kallikrein present in plasma is able to activate plasminogen (VanVliet, 1980).

The presence of enzymes that induce hemorrhage in *Crotalus* venoms has been observed in adult specimens of *C. durissus durissus* from Costa Rica, *C. d. cumanensis* and *C. vegrandis* from Venezuela (Gutierrez et al., 1991; Aguilar et al., 2001). Characterizing many of the *Crotalus* metalloproteases belonging to either hemorrhagic or fibrinolytic classes is difficult because many of these enzymes possess both activities. Sánchez (2004) showed that several *C. vegrandis* venom fractions presented fibrinolytic activity, but only a few were hemorrhagic. In the present study the hemorrhagic activity was also evaluated: Lagunetica venom showed the highest hemorrhagic activity, and Anzoátegui and Carrizales had weak hemorrhagic activity. Santa

Teresa, Guarenas, Aragua and Margarita lacked hemorrhagic activity. The comparison between the fibrinolytic and hemorrhagic activities of venoms demonstrated that Santa Teresa and Margarita had the lowest fibrinolytic activity without hemorrhagic activity. Lagunetica, and Carrizales with high, and Anzoátegui with moderate fibrinolytic activities showed hemorrhagic activity. In contrast, Aragua and Guarenas, which presented fibrinolytic activity, did not show hemorrhagic activity (Table 1).

Coagulopathy results from the activity of venom enzymes acting on fibrinogen (thrombin-like enzyme) or activating prothrombin and/or factor Xa that induce low levels of circulating fibrinogen and other clotting factors with important levels of fibrinogen degradation products, which certainly characterizes Viperidae bites in most regions of South America (Jorge and Ribeiro, 1992). By studying hemostatic alterations in snakebite victims, it was discovered that *C. durissus* envenoming is frequently associated with hemostatic disorders (Sano-Martins et al., 2001), which are almost certainly confirmed as thrombin-like enzymes activities. In Venezuela, *C.d. cumanensis* venom proteases with these activities have not been well classified and those that have an effect on coagulation have never been isolated.

In this study, the procoagulant activity of *C. d. cumanensis* venoms was also evaluated. This was first assayed by the amidolytic method, demonstrating the presence of direct thrombin-like activity in all the *C.d. cumanensis* venoms studied. The comparative analysis demonstrated that Santa Teresa, Guarenas , Aragua and Margarita venoms presented the highest coagulant activity and Carrizales, Lagunetica and Anzoátegui venoms, the lowest. These data could indicate variations in the composition and proportion of the active venom components (Table 3). The venoms did not present factor Xa-like activity.

Most Viperidae snake venoms have procoagulants (Eagle, 1937), or anticoagulants (Kruse and Dam, 1950) or both (Cheng and Ouyang, 1967). The coagulant method, in our study, showed that the purified fibrinogen was a better substrate than plasma, which evidenced that the physiological inhibitors present in this natural substratum (plasma) were effective against the thrombin-like proteases present in these crude venoms. Lagunetica and Anzoátegui venoms presented a low thrombin-like activity by this method. In addition, Carrizales venom was inactive on plasma and fibrinogen even at 100 μg/0.1 mL. It is plausible that the absence in Carrizales venom of thrombin-like activity, could be explained by the highest fibrinogenolytic activity observed in this venom. These results induced us to evaluate the possible fibrinogenolytic activity on purified fibrinogen.

Many snake venom proteases rapidly hydrolyze the fibrinogen Aα chain. Some of them hydrolyze the Bβ chain and rarely these proteases hydrolyze the γ chain. Two different classes of venom fibrin(ogen)-olytic enzymes were formerly recognized, the serine proteinases and metalloproteases (Swenson and Markland, 2005). Our results also demonstrated that Lagunetica and Anzoátegui venoms, which presented the lowest thrombin-like activity, and Carrizales venom that had no coagulant activity, induced a rapid (5 min) degradation of fibrinogen α chains and a slow (240 min) degradation of β chains. In this study, the γ chains appeared to be resistant to hydrolysis by the venoms. The intensity of the protein band corresponding to γ chain did not change over time, remaining unmodified after 24 h of incubation (data not shown). This probably represents the presence of α fibrinogenases in those venoms. Metalloprotease inhibitors abolished this effect, which suggest that this activity can be related to metalloprotease enzymes. The fibrino(geno)lytic enzymes of *C. d. cumanensis* could be of significant connotation because of their probable therapeutic importance in clinical syndromes in which fibrin deposition is concerned.

Geographical variations of snake venom is clearly documented in the literature (Johnson, 1968; Glenn et al., 1983; Glenn and Straight, 1989; Adame et al., 1990; Francischetti et al., 2000; Saravia et al., 2002; Dos-Santos et al., 2005; Salazar et al., 2006). Our observations also indicated that *Crotalus* venoms from diverse geographical areas presented differences in the size exclusion chromatographic and SDS-PAGE profiles (Figs. 4 and 5). The molecular exclusion chromatographic analysis showed that Anzoátegui, Carrizales and Lagunetica venoms had the largest proportion of high molecular mass components. Santa Teresa and Aragua had the highest proportion with intermediate molecular masses (I1 and I2 components). Anzoátegui venom presented all peaks in high concentration except the intermediate peaks. In general, the results evidenced molecular mass and concentrations differences among the seven venoms. Physicians encounter different clinical circumstances with each snakebite victim. Consequently, the importance of our study

would allow the production of more specific antivenoms, related to the snake's locations in order to obtain efficacious treatment of snakebite victims.

Our results represent important findings since the description of a fibrinolytic molecule, without hemorrhagic activity, in a Venezuelan *Crotalus*, can have valuable potential as a thrombolytic agent, for the treatment of patients with thrombosis disorders.

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