HAEMORRHAGIC, PROTEOLYTIC AND NEUROTOXIC ACTIVITIES PRODUCED BY DUVERNOY'S GLAND SECRETION FROM THE FALSE CORAL SNAKE (*Erythrolamprus bizona* Jan 1863) (SERPENTES:COLUBRIDAE)

Actividades hemorrágicas, proteolíticas y neurotóxicas producidas por la secreción de la glándula de Duvernoy de la falsa serpiente coral (*Erythrolamprus bizona* Jan 1863) (Serpentes: Colubridae)

Karel Lemoine and Alexis Rodríguez-Acosta

Universidad Central de Venezuela, Immunochemistry Section, Tropical Medicine Institute, Apartado 47423, Caracas 1041, Venezuela. E-mail: rodriguf@ucv.ve

RESUMEN

Muchas serpientes Colubridae producen secreciones orales tóxicas. En este trabajo se ha estudiado el veneno de la secreción de la glándula de Duvernoy colectado de especies opistoglifas (colmillos posteriores) de Coludridae Venezolanas. Se corrieron electroforesis de poliacrilamida al 20% (SDS-PAGE), a fin de separar y caracterizar las proteínas presentes en la secreción de la glándula de Duvernoy de la falsa coral *Erythrolamprus bizona.* El veneno mostró actividad proteolítica (gelatinasa). Para purificar parcialmente esta última actividad, se empleó una columna Mono Q (Bio-Rad, USA) de cromatografía de intercambio iónico. Para probar la actividad hemorrágica, de esta secreción de Duvernoy, se utilizaron embriones de pollo, piel y peritoneo de ratones, donde se demostró fuerte actividad hemorrágica. Al observar los síntomas neurológicos en ratones, ocasionados por la secreción de Duvernoy de *E. bizona,* se pudo constatar la intensa actividad neurotóxica de este veneno. En conclusión, la secreción de la glándula de Duvernoy de *E. bizona* mostró tener actividades proteolíticas, neurotóxicas y hemorrágicas.

Palabras clave: *Erythrolamprus bizona,* hemorragia, neurotoxinas, opistoglifas, secreción de Duvernoy.

ABSTRACT

Many colubrid snakes produce toxic oral secretions. Venom (Duvernoy´s gland secretion) collected from Venezuelan species of opisthoglyphous (rear-fanged) colubrid snakes has been studied. Electrophoresis 20% SDS-PAGE were run to separate and characterize the different proteins present in secretion from the Duvernoy gland in *Erythrolamprus bizona,* the false coral snake under study. The venom displayed proteolytic (gelatinase) activity. To partially purify this proteolytic activity a chromatography ionic exchange mono Q2 column was used. To test haemorrhagic activity *E. bizona* Duvernoy´s gland secretion on chicken embryos, mammalian (mice) skin and peritoneum were tested and haemorrhagic activity in both experiments was evident. Observing the symptoms of *E. bizona* Duvernoy´s gland secretion in mice, it was seen to produce neurotoxic activity since several disorders associated with neurotoxins were observed. In conclusion, *E. bizona* Duvernoy´s gland secretion showed proteolytic, haemorrhagic and neurotoxic activity.

Key words: *Erythrolamprus bizona,* haemorrhage, neurotoxins, opisthoglyphous, Duvernoy's gland secretion.

Recibido: 21 / 01 / 03. Aceptado: 07 / 07 / 03.

INTRODUCTION

Erythrolamprus bizona is a opisthoglyphous snake that feed on other snakes described from Costa Rica to Venezuela [15, 20, 25, 28]. This species is found from piedmont to moderate elevation of approximately 1000 m altitude living in tropical forest and premontano wooded area. Due their coloration is called "false coral snake". It has black head until the postoculars, with a white band behind parietals. The red rings have 7-10 scales with the apex black [20]. Nothing is known about the biochemistry and pharmacology of the Duvernoy´s gland secretion (DGS) of *E. bizona*. Searching published reports about this species DGS non references were found. Only snake biological descriptions emerge from the literature [9, 20].

The main aim of this study was to search pathological effects in mice caused by Colubridae *E. bizona* DGS providing new information and knowledge about this species venom.

MATERIALS AND METHODS

Animals

Albino Swiss NIH strain male mice of 18-22 g maintained under laboratory conditions, and obtained from the National Institute of Hygiene "Rafael Rangel" were used. The investigation complies with the bioethical norms taken from the guide "Principles of laboratory animal care" [2].

Snake captures were made on evening and crepuscular tours (without transect delimitations), at different geographical Venezuelan environments, with high emphasis, in those areas of interest for the study (Barlovento, Miranda state, Venezuela), where we had museum references of the opisthoglyphous snakes incidence.

Duvernoy's gland secretion

DGS from 3 snakes was collected through a 15 mL plastic centrifuge tube transversely cut and cover on the top with Parafilm. The snakes were obliged to bite the parafilm with its opisthoglyphous fangs. The DGS was milked with a micropipette. From each snake milking 0.2 mL secretion was approximately obtained [23].

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

Electrophoresis using a Dual Mini Slam kit AE-6450 (Atto Corporation, Tokyo, Japan) chamber was performed. SDS-PAGE was carried out [21], using 20% gels under reducing conditions. Molecular weight markers (Bio-Rad) were run in parallel and gels stained with Coomassie Blue R-250. *E. bizona* DGS samples to be analyzed were dissolved in a proportion of 1:1 in the solubiliser solution: 0.5 M Tris.HCl, pH 6.8, with 10% (w/v) SDS, 10% (v/v) ß-mercaptoethanol, 10% (v/v) glycerol and 0.05% (w/v) bromo phenol blue, and heated at 100°C for 10 minutes. The molecular weight was determined by Multi-Analyst TM/PC version 1.1 program (Bio-Rad).

Chromatographic analysis [1]

DGS (20 mgr) was diluted to 1.0 ml with 50 mM Tris-HCl, buffer pH 7.0 and exposed to a Mono Q2 column chromatography pre-equilibrated with the same buffer a 4°C. The column was washed with three column volumes of equilibrating buffer at a flow rate of 1.0 ml/min. DGS proteins were eluted with a gradient of 0-1 M NaCl dissolve in 50mM Tris-HCl pH 7-9. The fraction size was 0.5 ml. Elution of protein was monitored at 280 nm. The eluting peak tops were tested for proteolytic activity.

Haemorrhagic activity tested on chicken embryos

Embryonic hen eggs incubated at 37°C for 5 days were cleaned with 70% alcohol and the embryos were extracted breaking the eggshells. Embryos were put on a Petri dishes and incubated at 37°C for 3 hours.

Circles of filter paper Watmann N° 2 of 3 mm diameter were impregnated with 3 µL (5.3 µg) of the *E. bizona* DGS and applied to the chicken embryo vitelin vein [10, 24, 29]. Circles soaked with 3 µL (0.75 µg) of *Bothrops venezuelensis* venom as positive control and sodden with saline solution as negative control were also used.

Determination of haemorrhagic activity on skin

DGS haemorrhagic activity was determined by a modification of Kondo´s test [14, 19]. *E. bizona* DGS containing 5.25 µg/100 µL were injected intradermal into the abdominal skin of four male NIH Swiss albino mice. The skins were removed 6 hours after and the haemorrhagic spots diameters on the inside surfaces were measured [17]. Two diameters were achieve for the spot of haemorrhage by measuring the longest diameter of the spot and the diameter perpendicular to the first measurement. A minimal haemorrhagic dose (MHD) was taken as the end point and defined as that concentration of venom resulting in a 10 mm haemorrhagic spot [3]. *B. venezuelensis* venom (15 mg/Kg of weight mouse) and saline solution were used as positive and negative controls respectively.

Determination of haemorrhagic activity on peritoneum

One hundred microlitres of *E. bizona* DGS containing 2.62 µg were injected intraperitoneally into four male NIH Swiss albino mice. *B. venezuelensis* venom (15 mg/Kg of weight mouse) and saline solution were used as positive and negative controls respectively.

Neurotoxic activity

To determine the neurological signs and symptoms which may be produced by *E. bizona* DGS six mice were subcutaneously injected with 100 µL (262.5 mg/Kg of weight mouse). Neurological symptoms: Dyspnea, paralysis and cyanoses were clinical observed.

Gelatinase Assay

An assay modified [18] was used to test gelatinase activity of *E. bizona* DGS. The X-ray film was washed down with distilled water and incubated at 37°C for 45 min. After incubation, the film was placed to dry completely and 25 microlitres of crude DGS as well as the ion chromatography obtained fractions on dilutions from 1 to 128 (4.9 mg/mL solution) were placed on a Kodak X-OMAT scientific imaging film with gelatine coating.

Hydrolysis of gelatine on the X-ray film was determined after a 4 hr incubation at 37°C in a humid incubator by washing the film with distilled water.

Serial dilutions were performed to determine the minimum amount of DGS required to cause a clear spot on the X-ray film. The titer is defined as the reciprocal of the highest dilution that caused a clear spot on the X-ray film. The specific gelatinase activity was calculated by dividing the titer by the amount of protein (µg) applied on the film. The assay was repeated three times.

RESULTS

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis

SDS-PAGE proteins presented in *E. bizona* DGS is showed in FIG. 1. The relative masses were determined using the Multi-Analyst TM/PC version 1.1 (Bio-Rad) program.

Ionic interchange chromatography

E. bizona. DGS run in a Mono Q2 column chromatography produced 11 peaks (FIG. 2). The eluting peak tops were tested for proteolytic activity.

Haemorrhagic activity tested on chicken embryos

FIG. 3 showed the haemorrhagic activity of *E. bizona* DGS on the chicken embryo vitelin vein. An obvious vascular blood extravasation was observed. Saline solution negative and *B. venezuelensis* venom positive controls were also demonstrated.

Determination of haemorrhagic activity on skin

E. bizona DGS had haemorrhagic activity when tested by intradermal injections in mice, *B. venezuelensis* positive control and negative control are also showed (FIG. 4).

Determination of haemorrhagic activity on peritoneum

All mice intraperitoneally injected with *E. bizona* DGS showed severe haemorrhagic activity (FIG. 5). Saline solution negative control and *B. venezuelensis* venom positive control were also determined.

Proteolytic (gelatinase) activity

The peaks obtained by chromatography and the secretion from crude *E. bizona* DGS were set on X-ray film showing the proteolytic activity of the crude DGS until dilutions of 1: 128. The chromatography corresponding peaks at P6, P7, P8, P9 and P10 demonstrated proteolytic activity. The peak P8 until dilutions of 1:8 and P6, P7, P9 and P10 until dilutions 1:4.

Neurotoxic activity

E. bizona DGS neurotoxic activity was established by the neurological clinical manifestations observed in all mice subcutaneously injected with this DGS (TABLE I).

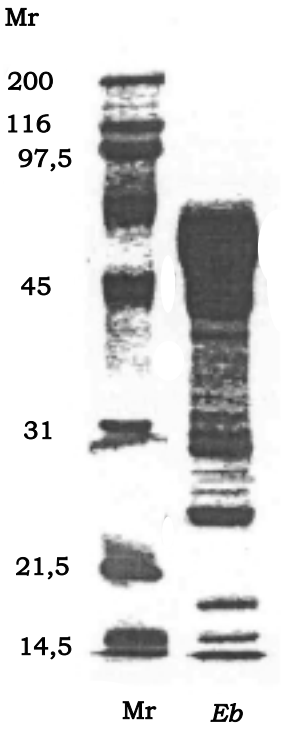


FIGURE 1. *ERYTHROLAMPRUS BIZONA* DUVERNOY´S GLAND SECRETION RUN IN A 20% POLYACRYLAMIDE GEL ELECTROPHORESES (SDS-PAGE).

DISCUSSION

E. bizona DGS showed a considerable potential basis of novel biological substances. Despite the lack of described human cases, caution should be exercised when manipulating these snakes, and bitten people should be closely observed for the potential development of bleeding and coagulopathological process, proteolysis and neurological symptoms using the mice model that it has been described in this work.

Saliva of some “non-venomous” colubrids have been reported to have toxic secretions that are capable of causing severe symptoms [4-8, 9, 16, 30].

In Venezuela, only some authors [27] have described a number of DGS proteins and biological characteristics in a Venezuelan colubrid snake (*Philodryas viridissimus*). Eight bands of proteins were described and they were compared with proteins expressed by *Crotalus* (*C. durissus cumanensis*, *C. durissus ruruima*, *C. vegrandis, C. pifanorum* and *C. unicolor*) venom. In the present work, *E. bizona* DGS peaks partially purified presented important proteolytic activity (1:8 dilutions) on gelatine gels. Colubrid *T. b. lambda* DGS may lack gelatine-degrading proteases being significant in *E. bizona.* Venoms from several colubrid species such as *Trimorphodon biscutatus lambda* showed proteolytic activity [12] when assayed with casein substrates. Most caseinolytic and gelati-

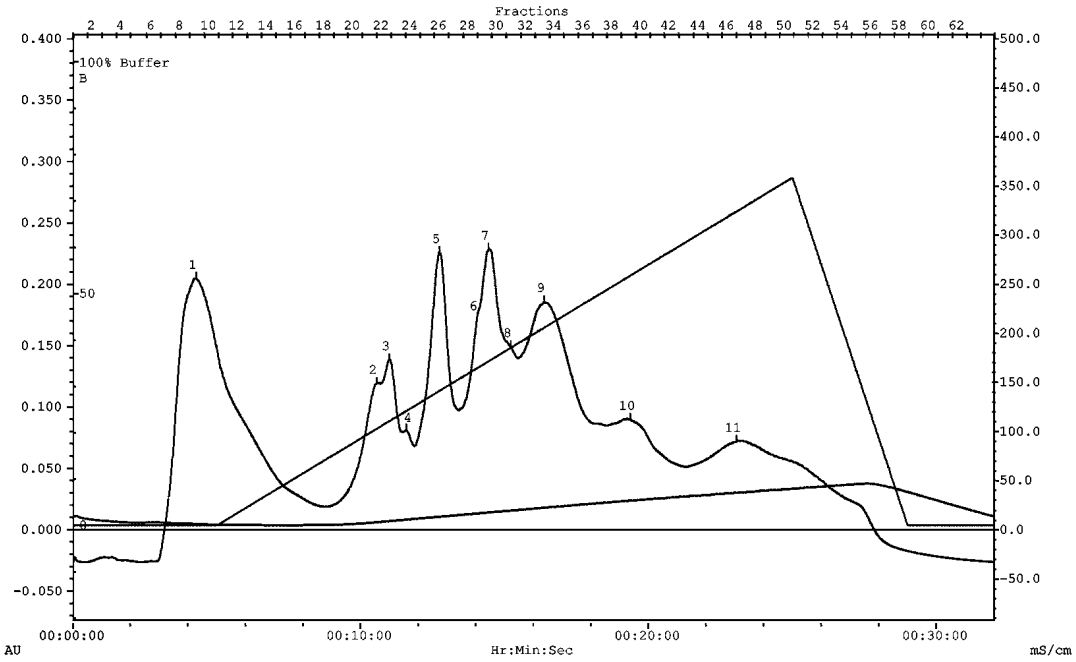
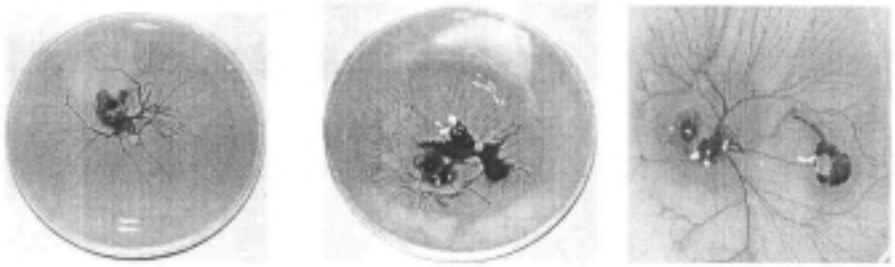
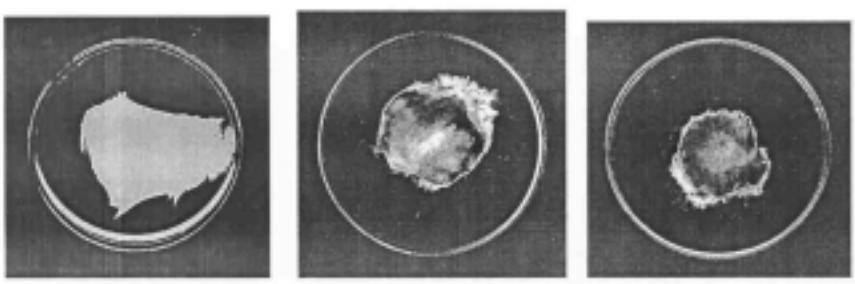


FIGURE 2. SEPARATION OF *ERYTHROLAMPRUS BIZONA* DUVERNOY´S GLAND SECRETION FRACTIONS BY ION-EXCHANGE CHROMATOGRAPHY ON A MONOQ2 COLUMN. PEAKS 6,7,8,9 AND 10 SHOWED PROTEOLYTIC ACTIVITY.



A **B** **C**

FIGURE 3: *ERYTHROLAMPRUS BIZONA* DUVERNOY´S GLAND SECRETION HAEMORRHAGIC ACTIVITY ON CHICKEN EMBRYOS. A: NEGATIVE CONTROL (SALINE SOLUTION); B: POSITIVE CONTROL (*BOTHROPS VENEZUELENSIS* VENOM); C: *ERYTHROLAMPRUS BIZONA* DUVERNOY´S GLAND SECRETION.



A **B** **C**

FIGURE 4. *ERYTHROLAMPRUS BIZONA* DUVERNOY´S GLAND SECRETION HAEMORRHAGIC ACTIVITY ON MICE SKIN. A: NEGATIVE CONTROL (SALINE SOLUTION); B: POSITIVE CONTROL (*BOTHROPS VENEZUELENSIS* VENOM); C: *ERYTHROLAMPRUS BIZONA* DUVERNOY´S GLAND SECRETION.

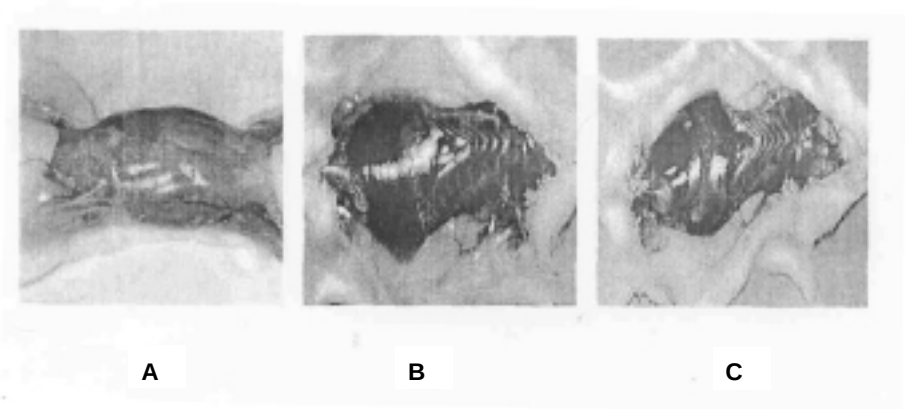


FIGURE 5. *ERYTHROLAMPRUS BIZONA* DUVERNOY´S GLAND SECRETION HAEMORRHAGIC ACTIVITY IN PERITONEUM. A: NEGATIVE CONTROL (SALINE SOLUTION); B: POSITIVE CONTROL (*BOTHROPS VENEZUELENSIS* VENOM); C: *ERYTHROLAMPRUS BIZONA* DUVERNOY´S GLAND SECRETION .

TABLE I
THE RESULTS CORRESPOND TO A POOL OF NEUROTOXIC SIGNS AND SYMPTOMS FROM MICE INTRAPERITONEALLY INOCULATED WITH *ERYTHROLAMPRUS BIZONA* DUVERNOY´S GLAND SECRETION

Time (min)	Dyspnoea	Cyanoses	Posterior limbs paralysis	Flaccid paralysis	Urinary sphincter relaxation	Pilo erection	Mice Death
2	2s						
4	6s	2s					
12	1s,4s		2s	2s			
16		4s	6s	6s			2s
18	3s,5s	3s	1s,5s				6s
30				4s		1s	
86				3s		3s	
96				1s		5s	
104				5s		4s	1s,3s,4s
114							5s

Only six mice were used to test neurotoxical symptoms. Each mouse is represented by a number from 1s to 6s.

nases from viperid snake venoms are metalloproteases [1]. Proteolytic activity of *E. bizona* supporting tissue digestion was unknown in the literature.

Authors [29] have used chicken embryos to test haemorrhagic activity and antivenin efficiency on Viperidae and Elapidae snakes. Navarrete et al. [24] were the first authors testing haemorrhagic activity of colubrid venom on chicken embryos. This is the first time that this method testing haemorrhagic activity on vitelin veins of these embryos in *E. bizona* DGS has been used. In addition, in the murine model, the skin and peritoneum haemorrhagic damages occasioned by *E. bizona* DGS activity, confirmed that this colubrid has a very strong haemorrhagic action on mammal tissues. Other [22] had already described large haemorrhagic areas into mice internal organs produced by the colubrid *Leptodeira annulata* DGS.

Testing neurological activity by clinical symptoms in mice injected with *E. bizona* DGS, several neurotoxic symptoms such as spastic and/or flaccid paralysis were noticed. Non bibliographical references were found describing neurological alterations occasioned by this DGS. As far as known this is the first time that these signs and symptoms produced by this colubrid snake DGS has been described. The most notable activities were the dyspnea, posterior limbs paralysis, which appeared 2 and 12 min respectively, after DGS injection. Flaccid paralysis appeared 12 min after injection and all animals died before 114 min, probably by respiratory paralysis. Only some authors [11] had described colubrid neurotoxic alterations, that fractions causing these symptoms probably involve post or presynaptic neurotoxins, and they are responsible for the flaccid and/or spastic paralysis [13, 26]. The presence of a Mr 14 kDa

band found in *E. bizona* DGS (FIG. 1) similar to the already described Mr 14 kDa of phospholipase A2 neurotoxic (from *Viperidae*) may possibly be a similar toxin, which could elucidate the neurotoxic activity (flaccid paralysis) described in this work.

CONCLUSIONS

Opisthoglyphous colubrids such as *E. bizona* represent a inestimable source of unknown venoms which give good reason for further research. The unexpected results here obtained (haemorrhagic, neurotoxic and proteolytic activities) were much more encouraging that predictable. The toxic possibilities that these species have for a long time were forgotten by most of toxin researchers.

BIBLIOGRAFIC REFERENCES

[1] AGUILAR, I.; GIRÓN, M.E.; RODRÍGUEZ-ACOSTA, A. Purification and characterisation of a haemorrhagic fraction from the venom of the Uracoan rattlesnake *Crotalus vegrandis*. **Biochem.Biophys.Acta.** 1548: 57-65. 2001.

[2] NATIONAL INSTITUTE OF HEALTH. **Principles of laboratory animal care**, USA. Pub. 85 Nº 23: 1-112. 1985.

[3] ASSAKURA, M.; REICHL, P.; MANDELBAUM, F.R. Isolation and characterization of five fibrin(ogen)olytic enzymes from the venom of *Philodryas olfersii* (Green snake). **Toxicon**. 32: 819-831. 1994.

[4] COOK, D.G. A case of envenomation by the neotropical colubrid snake, *Stenorrhina freminvillei*. **Toxicon**. 22: 823-827. 1984.

[5] COWLES, R.B.; BOGERT. C.M. Observations on the California lyre snake, *Trimorphodon vandenburghi*, Klauber, with notes on the effectiveness of its venom. **Copeia**. 1935: 80. 1935.

[6] DE ARAUJO, M.E.; DOS SANTOS, A.C. Cases of human envenoming caused by *Philodryas olfersii* and *Philodryas patagoniensis* (Serpentes: Colubridae). **Rev. Soc. Bras. Med. Trop.** 30: 517-519. 1997.

[7] DE LEMA, T. Relato de um envenenamento por uma cobra nao venenosa. **Natur. Rev**. 4: 62-63. 1978.

[8] DE LISLE, H.F. Venomous colubrid snakes. **Bull. Chic. Herpetol. Soc.** 17: 1-17.1982.

[9] DE LISLE, H.F. *Boiga cyanea* (Green Cat-eye Snake): Envenomation. **Herpetol. Rev**. 15: 112. 1984.

[10] DUNN, B.E.; BOONE, M.A. Growth Of The Chick Embryo *In Vitro.* **Poultry Science**. 55: 1067-1071. 1976.

[11] FONTANA, M.D.; HELENO, M.G.; VITAL-BRAZIL, O. Mode of action of Duvernoy´s gland extracts from the colubrid *Dryadophis bifossatus* in the chick biventer cervicis nerve-muscle preparation **Toxicon**. 34: 1187-1190. 1996.

[12] FOX, J.W.; LONG, C. The ADAMs/MDC family of proteins and their relationships to the snake venom metalloproteinases. In: **Enzymes from snake venom**. Eds. Bailey, G.S. Alaken Press, Fort Collins, CO. 151-178 pp.1998.

[13] GRENARD, S. **Medical Herpetology**. Reptile and Amphibian Magazine, Pottsville PA, USA. 1-139.pp. 1994.

[14] GUTIÉRREZ, J.M.; LEÓN, G.; ROJAS, G.; LOMONTE, B.; RUVACADO, A.; CHAVES, F. Neutralization of local tissue damage induced by *Bothrops asper* (terciopelo) snake venom. **Toxicon**. 36: 1529-38. 1998.

[15] HAYES, M.P.; POUNDS, J.A.; TIMMERMAN, W.W. An annotated list and guide to the amphibians and reptiles of Monteverde, Costa Rica. **SSAR. Herp. Circ.** 17: 1-67. 1998.

[16] HILL, R.E.; MCKESSY, S.P. Characterization of venom (Duvernoy´s secretion) from twelve species of colubrid snakes and partial sequence of four venom proteins. **Toxicon.** 38: 1663-1687. 2000.

[17] HUANG, S.Y.; PÉREZ, J.C. Comparative study on hemorrhagic and proteolytic activities of snake venoms. **Toxicon**. 18: 421-426. 1980.

[18] HUANG, S.Y.; PÉREZ, J.C. A comparative electron microscopic study of myonecrosis induced by *Crotalus atrox* (Western Diamondback Rattlesnake) in gray woodrats and mice. **Toxicon**. 20: 443-449. 1982.

[19] KONDO, H.; KONDO, S.; IKEZAWA, H.; MURATA, R.; OHSAKA, A. Studies on the quantitative method for determination of hemorrhagic activity of habu snake venom. **Jap.J. Med. Sci. Biol**. 13: 43-49. 1960.

[20] LANCINI, A.R. **Serpientes de Venezuela**. Ediciones E. Armitano, Caracas, Venezuela. 1-262 pp. 1979.

[21] LAEMMLI, U.K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4 . **Nature.** 227: 680-685. 1970.

[22] MEBS, D. Analysis of *Leptodeira annulata* venom. **Herpetologica**. 24:338-355. 1968.

[23] MUNEKIYO, S.M.; MACKESSY, S.P. Effects of temperature and storage conditions on the electrophoretic, toxic and enzymatic stability of venom components. **Comp.Biochem.Physiol.** 119B: 119-127. 1998.

[24] NAVARRETE, L.F.; LEMOINE, K.; RODRÍGUEZ-ACOSTA, A. Is the opistoglyph *Clelia clelia* Duvernoy´s gland secretion haemorrhagic in humans? **Act.Biol.Ven.** 19: 19-23. 1999.

[25] PETERS, J. A.; OREJAS-MIRANDA, B. Catalogue of the Neotropical Squamata. Part I: Snakes. **Bull. U.S. Natl. Mus.** 297: 1-347. 1970.

[26] RODRÍGUEZ-ACOSTA, A.; MONDOLFI, A.; ORIHUELA, A.; AGUILAR, M. **¿Qué hacer frente a un accidente ofídico?** Primera Edición. Venediciones C.A, Caracas, Venezuela. 1-108 pp. 1995.

[27] RODRÍGUEZ-ACOSTA, A.; GIRÓN, M.E.; AGUILAR, I.; FUENTES, O. A case of envenomation by a "non venomous" snake (*Philodryas viridissimus*) and comparison between this snake Duvernoy´s gland secretion and northern South America rattlesnakes venoms. **Arch.Ven.Med.Trop**. 1: 29-32. 1997.

[28] SAVAGE, J. M.; VILLA, J. An Introduction to the Herpetofauna of Costa Rica. **Soc. Stud. Amphib. Rept. Contrib. Herpetol.** 3: 1-207. 1986.

[29] SELLS, P.G.; RICHARDS, A.M.; LAING, G.D.; THEAKSTON, R.D.G. The use of hens´eggs an alternative to the conventional *in vivo* rodent assay for antidotes to haemorrhagic venoms. **Toxicon**. 35: 1413-1421. 1997.

[30] WEINSTEIN, S.A.; KARDONG, K.V. Properties of Duvernoy´s secretions from opisthoglyphous and aglyphous colubrid snakes. **Toxicon**. 32: 1161-1185. 1994.