Pergamon

PII: S0041-0101(96)00174-2

Toxicon, Vol. 35, No. 5, pp. 671–678, 1997

Printed in Great Britain
0041-0101/97 $17.00 + 0.00

VENOM YIELDS FROM SEVERAL SPECIES OF COLUBRID SNAKES AND DIFFERENTIAL EFFECTS OF KETAMINE

ROBERT E. HILL and STEPHEN P. MACKESSY*

Department of Biological Sciences, 501 20th Street, University of Northern Colorado, Greeley, CO 80639, U.S.A.

(*Received* 26 *June* 1996; *accepted* 9 *September* 1996)

R. E. Hill and S. P. Mackessy. Venom yields from several species of colubrid snakes and differential effects of ketamine. *Toxicon* **35,** 671–678, 1997.—The composition of rear-fanged colubrid snake venoms is largely unknown due primarily to the difficulty involved in venom collection. Several different methods have been used to maximize the yield of Duvernoy's secretions. The method proposed by Rosenberg in 1992, which includes the use of ketamine hydrochloride anesthetic and pilocarpine to induce Duvernoy's glands secretion, was used in the present study to collect venom from eight species of colubrids. Protein concentrations, using a dye-binding microassay technique, were determined for the venoms collected. Average protein concentrations ranged from 49.8 to 96.4%. Most yields (dry weight/snake) obtained from specimens in this study were significantly greater than yields previously reported. There was a wide range of effects that occurred due to the ketamine injections; however, all snakes recovered from the effects of the ketamine hydrochloride/pilocarpine with no apparent ill effects. Recommended doses of ketamine hydrochloride have thus been adjusted, depending on previous reactions to the drug. The use of ketamine/pilocarpine in the collection of Duvernoy's secretion has proven to be highly effective in increasing yields. Some caution should be observed when administering ketamine to various species of colubrids, as effects do not necessarily scale to body mass. © 1997 Elsevier Science Ltd

INTRODUCTION

Animal venoms are rich sources of biologically active components with many uses in medicine and biochemistry (e.g. Shier and Mebs, 1990; Pirkle and Markland, 1988), and rear-fanged colubrid snake venoms represent a largely unknown source of potentially novel compounds. Additionally, the distinct evolutionary history of colubrids and the extreme specialization of diet of some species further suggest that colubrid venoms deserve further

*Author to whom correspondence should be addressed.

characterization (Weinstein and Kardong, 1994). It has been suggested that nearly half of the species in this large family of snakes produce a venom to some degree (Gans, 1978), and at least three colubrids have been reported to cause human fatalities (Ogawa and Sawai, 1986; FitzSimons and Smith, 1958; Pope, 1958). As interactions between humans and potentially dangerous colubrids increase (through human encroachment into remote areas, the pet industry and introductions of colubrids to new areas), the need to gain a better understanding of colubrid venoms has also increased.

The extraction of venom produced in the Duvernoy's gland of colubrid snakes historically has been a difficult process because of the small amounts of venom secreted and released at the base of enlarged teeth at the rear of the maxillary bone. Because these teeth are not hollow (they may be paired or grooved), venom typically must be collected at the base of these fangs. Several different methods have been used to attempt to maximize the yield of Duvernoy's secretion collections, with various degrees of success. These methods of collection have included maceration of the Duvernoy's glands from dead colubrids (Kornalik *et al.*, 1978; Robertson and Delpierre, 1969; McAlister, 1963), collection of venom with washable absorbants such as membranes or foam (Theakston *et al.*, 1979), and collection at the base of the rear fangs via micropipette aspiration (Glenn *et al.*, 1992; Vest, 1981). Problems with these various methods can be numerous. With some methods, such as using a membrane for collection, it is difficult to isolate saliva-free venom, complicating subsequent analysis (e.g. Glenn *et al.*, 1992). Venom obtained from microaspiration could be quantified, but yields from small species are typically quite low; for example, the average yield of Duvernoy's secretions in a study of *Thamnophis elegans vagrans* (wandering garter snake) was 0.71 μl/snake, with an average dry weight of 57 μg/snake (average of 80.3 μg/μl of dry weight liquid yield; Vest, 1981). Vest (1988) reported an average yield of 4.22 μl for *Hypsiglena torquata texana* (Texas night snake) with an average dry yield of 400 μg (average of 95.0 μg/μl of dry yield per liquid yield). For larger rear-fanged colubrids such as *Boiga irregularis* (brown tree snake), with body weights > 900 g, yields of 50–215 μl have been reported (Chiszar *et al.*, 1992). No other reported data exist for venom yields from other species of colubrids.

Protein concentrations of colubrid venoms (Duvernoy's secretions) can vary between species, individuals of the same species, and perhaps between methods of collection. Venom collected from *B. irregularis* has ranged from 22.8% protein of lyophilized samples (Vest *et al.*, 1991) to 100% protein (Weinstein *et al.*, 1991, 1993). Vest (1981) reported a protein concentration of 55.5% for Duvernoy's secretion from *T. e. vagrans*, and a protein concentration of 77% was reported for *H. t. texana* (Vest, 1988). Collection methods that yield large amounts of venom with little saliva contamination should be high in protein, because Duvernoy's secretion tends to have a higher protein concentration than saliva. Protein concentrations of venoms from most species analysed in the present study have not been previously reported.

An extraction method that would allow for increased venom yields sufficient for detailed analyses of the samples is desirable. In addition to increasing venom yields, a technique that allows for an increased efficiency of extraction from very small specimens and easier handling of large specimens would be advantageous. A recent method, which involves ketamine anesthesia followed by parasympathetic stimulation of the Duvernoy's gland with pilocarpine (Rosenberg, 1992), appeared most promising. The present study utilizes this method with some modification and demonstrates that high to very high yields can be obtained from both small and large opisthoglyphous colubrids. Additionally, since

snakes are not killed, repeated extractions from the same snake can be made without apparent ill effects to the snake.

MATERIALS AND METHODS

Reagents

Protein concentration reagent was obtained from BioRad (U.S.A.). Ketamine–HCl was a product of Fort Dodge Laboratories (U.S.A.) All other reagents (analytical grade) were obtained from Sigma Biochemical Corp. (U.S.A.).

Snakes

The following species were used in this study: *Boiga irregularis*, *Diadophis punctatus regalis* (regal ringneck snake), *Heterodon nasicus kennerlyi* (Mexican hognose snake), *H. n. nasicus* (western hognose snake), *Hydrodynastes gigas* (false water cobra), *Hypsiglena torquata*, *Tantilla nigriceps* (blackhead snake), *T. e. vagrans*, *Trimorphodon biscutatus lambda* (Sonoran lyre snake) and an aglyphous snake, *Pituophis melanoleuca sayi* (bullsnake; for saliva control). Native colubrid snakes were collected in Arizona (permit no. MCKSY000221 to SPM) and Colorado (permit no. 95-0456 to SPM). Two specimens of *H. gigas* were on loan from Dr Samuel S. Sweet. Permission to extract venom from two *B. irregularis* was granted by Dr David Chiszar. One specimen of *T. b. lambda* was on loan from Dr Wade Sherbrooke.

Extraction of Duvernoy's secretions

The method of extraction was essentially identical for all snakes reported, and is based on the methodology for Duvernoy's secretion collection reported by Rosenberg (1992). However, instead of initially anesthetizing the subjects with halothane followed by ketamine hydrochloride, subjects in this study were anesthetized with only ketamine in all but two cases (Table 1). Based on preliminary experiments, doses of ketamine were usually well below 60 μg/g body weight. Venom samples were collected via 50 μl micropipettes placed around the enlarged rear tooth and within the fang sheath when present. The volume was estimated and venom was transferred to glass vials, frozen and lyophilized, stored frozen with desiccant and weighed out and resolubilized as required.

Protein concentration assay

The protein concentration of the samples was determined by the method of Bradford (1976) as modified by BioRad. Venom samples were prepared at apparent concentrations of either 2.0 or 4.0 μg/μl, and 5 μl of sample was then added to 795 μl of $d\mathrm{H_2O}$. Bovine gamma globulin protein standards of 5, 10, 15, 20 and 30 μg were used, also with a total volume of 800 μl. BioRad dye reagent (200 μl) was then added to each assay and standard tube, gently vortexed and allowed to stand at room temperature for 5–10 min. Absorbance was read at 595 nm for all tubes, and the averages of the blank tubes were subtracted from the averages of the standard and assay tubes.

RESULTS

Differential effects of ketamine

There was a wide range of responses from the snakes anesthetized with ketamine, and doses were adjusted accordingly (Table 1). The effect of the drug on a subject varied with the particular species, the size of the snake and whether ketamine had been administered to the subject during a previous extraction. Some subjects resisted the effects of the ketamine; this primarily occurred with several specimens of *H. n. nasicus* and one specimen of *D. punctatus*. None of the subjects had been administered ketamine prior to the study, and while they became somewhat subdued they never became sufficiently anesthetized to allow significant venom extraction. Other specimens of *D. punctatus* and *H. nasicus* that had been administered ketamine previously (typically 4–6 weeks earlier) became anesthetized rapidly. Ketamine (at 30 μg/g) used in conjunction with xylazine (at 1 μg/g body weight) was used on one specimen of *B. irregularis*. The recovery rate for this snake

Table 1. Doses, yields and protein content of venoms extracted from adult colubrid snakes

Species	*N*	Doses (μg/g): Keta; Pilo	μl Venom yield	mg Venom dry yield $\bar{x}$ (range)	% Protein in venom $\bar{x}$ (range)	% Protein in saliva $\bar{x}$ (range)
Boiga irregularis	2	50; 7.5*	370 (290–450)	7.7 (2.4–13.0)	79.4 (58.8–100.0)	ND
Diadophis punctatus regalis	4	45; 6.0–7.5	10 (5–20)	2.88†	100‡	ND
Heterodon nasicus kennerlyi	2	30; 7.5	15 (10–20)	—§	55.8	ND
Heterodon n. nasicus	2	45–60; 7.5	24 (20–28)	—§	73.2 (64.3–84.0)	21.9
Hydrodynastes gigas	11	15–60; 5.0–7.0	423 (110–840)	7.31 (2.3–15.2)	67.1 (31.8–97.8)	30.9 (13.5–48)
Hypsiglena torquata	8	40–60; 6.0–7.5	12 (5–30)	0.53 (0.28–1.05)	49.8 (21.7–100)	ND
Tantilla nigriceps nigriceps	2	25–45; 7.5	13 (10–15)	0.08 (0.05–0.10)	95.6‖	ND
Thamnophis elegans vagrans	10	60; 7.5¶	23 (10–45)	0.39 (0.10–0.46)	51.6 (32.7–84.6)	8.7 (3.3–12.6)
Trimorphodon biscutatus lambda	3	40; 7.5	130 (125–135)	6.34 (4.98–7.70)	98.2 (96.4–100)	ND
Pituophis melanoleuca	1	50; 7.5	~ 50	1.80	—	8.2

*One specimen was administered xylazine in addition to ketamine.
†Two samples were pooled; all other samples were too small to weigh accurately.
‡Owing to small sample size this value is an estimate. The pooled sample was also 100%.
§Samples were too small to weigh accurately.
‖Protein concentration was determined for only one sample.
¶One specimen was administered telazol instead of ketamine.
ND, Not determined.

Table 2. Relative yields of colubrid venoms: a comparison of the present method with other collection methods

Species	Average venom dry yields per snake (mg)	Reference
Boiga irregularis	13.0 and 2.4	This study
Boiga irregularis	10.8	Chiszar *et al.* (1992)
Hydrodynastes gigas	7.31	This study
Hydrodynastes gigas	1.3*	Glenn *et al.* (1992)
Hypsiglena torquata	0.53	This study
Hypsiglena torquata	0.40	Vest (1988)
Thamnophis elegans vagrans	0.39	This study
Thamnophis elegans vagrans	0.06	Vest (1981)

*This is a maximum value for venom collected from one gland only.

was nearly 2 weeks, much longer than the recovery rate for the other specimen of *B. irregularis* which was administered only ketamine (and recovered overnight).

Venom and saliva yields

There was a wide variety of venom and saliva yields, primarily dependent on species and size of the subjects (Table 1). The largest liquid yields obtained were from *H. gigas*, with the two largest yields being 650 μl from one specimen and 840 μl from another (Table 1). Other large snakes, such as *B. irregularis*, produced yields of 290–450 μl (Table 1). Significant yields from small snakes, including *D. p. regalis*, *H. n. kennerlyi*, *H. n. nasicus*, *H. torquata*, *T. nigriceps*, *T. e. vagrans* and *T. b. lambda*, were also obtained using this method. Approximately 50 μl of saliva was collected from *P. melanoleuca*, an aglyphous snake, for use as a non-contaminated saliva control. Using the present method, venom dry yields per snake were equivalent to or greater than published yields (Table 2).

Protein concentrations

High protein concentrations reflect the successful collection of venom, which characteristically contains several to many protein components; saliva is typically low in protein concentration and contains few protein components. The highest average protein concentrations in this study were in venoms from *D. p. regalis* at 100%, *T. nigriceps* at 95.6% and *T. b. lambda* at 100% (Table 1). Venoms collected from *B. irregularis* and *H. n. nasicus* also contained high protein concentrations (79.4% and 73.2%, respectively; Table 1). Saliva collected from several rear-fanged colubrids had protein concentrations below 31%, typically much lower (Table 1); however, it is possible that these samples were slightly contaminated with venom, which would tend to inflate values. Saliva collected from *P. melanoleuca*, an aglyphous colubrid, had a protein concentration of 8.2% (Table 1), which was significantly lower than the protein concentrations of any Duvernoy's secretions collected.

DISCUSSION

In the study performed by Rosenberg (1992), ketamine was administered at 60 μg/g body weight, and this concentration was used for the first extractions performed in this study. This amount proved to be excessive for most specimens, and dosages were decreased according to species and effects noted in different individuals. Adverse initial reactions to the anesthetic seemed to depend not only on species, but also on whether or not the

specimen had been administered ketamine previously. Some snakes were refractive to the effects of the anesthetic the first time ketamine was administered, but were subdued more readily and at lower dosages during subsequent extractions; other species, primarily *H. gigas*, seemed to be highly sensitive to ketamine and reacted violently every time it was administered. Neither specimen of *H. gigas* reacted when the needle was inserted subcutaneously, but both reacted with violent thrashing when the ketamine was administered during the first extractions. The snakes were anesthetized rapidly and did not recover for several days. Owing to the rapid onset of the anesthesia and prolonged effect that it had, dosage was cut from 60 μg/g to 15 μg/g. Although it took somewhat longer to anesthetize the snakes with this reduced dosage, the snakes recovered in a much shorter time, and adverse reactions diminished significantly. The recovery time for all specimens seemed to be shorter on subsequent injections, probably because of induction effects. Based on the highly prolonged effect on one specimen of *B. irregularis*, the combined use of xylazine and ketamine should be avoided.

Most of the snakes in this study showed little or no adverse reaction to ketamine. They were readily anesthetized by the drug and recovered fully within 6–24 hr. With the exception of some excess salivation, none of the specimens used in this study showed adverse reactions to the administration of the pilocarpine. To date, we have extracted a single individual up to four times at 6 week intervals with no detectable changes in activity, feeding patterns or general health. It should be noted that in general, ketamine dosages per gram body weight should be decreased for larger animals, and doses of approximately 15–20 μg/g should be used with snakes over 500–1000 g. Garter snakes seemed somewhat refractive to the anesthetic, and doses of 60 μg/g are recommended for natricine snakes of small to moderate size. In addition, since the snakes tend to lose moderate amounts of water, it is important to ensure that they are rehydrated after recovery from anesthetic.

The use of ketamine anesthetic and pilocarpine to induce secretion of the Duvernoy's glands greatly increased venom volume yields for both large and small colubrids. The extractions from *H. gigas* were the largest yields reported for the species, and the yield for *Trimorphodon biscutatus lambda* in this study (150 μl; Table 1) was at least three times higher than yields previously reported for this species (7–48 μl; Minton and Weinstein, 1987). Yields for *T. elegans vagrans* were ten to 45 times greater (Vest, 1981), and yields for *H. t. texana* were up to seven times greater than those previously reported (Vest, 1988). Dry weight protein yields were equivalent to or significantly greater than those previously reported (Table 2).

Protein concentrations of the lyophilized venoms are similar to protein concentrations previously reported using other collection methods; overall yields, however, were greatly increased. The protein concentrations obtained in this study for venom from *T. e. vagrans* were comparable to the percentage published by Vest (1981), and in two cases exceeded the figure of 77% reported in that study. Protein concentrations for some species in the study, including *H. nasicus* and *T. nigriceps*, have not been reported in previous studies. The protein concentration for the control (*P. melanoleuca* saliva; 8.2%) was significantly lower than values for all venoms reported in this study. The primary purpose for including this control was to ensure saliva collection with no chance of venom contamination. This result was expected because this species lacks a Duvernoy's gland but has prominent salivary glands; since saliva is largely composed of mucopolysaccharides, it should lack most of the protein components found in venoms. It is virtually impossible to ensure that colubrid venoms collected by this or any other method are entirely free of saliva contamination, but the fact that all of the venoms collected in this study had high protein

concentrations indicates that these secretions are indeed made up primarily of serous venom components.

It should be noted that for those species where liquid and dry yield data have been reported, the use of ketamine and pilocarpine results in a significantly larger volume yield of a more dilute secretion (see Table 2). It has been noted previously (Rosenberg, 1992; Rosenberg *et al.*, 1985) that the secretion obtained was less concentrated than that obtained without parasympathetic stimulation, and secretion composition does not seem to be affected (Rosenberg, 1992; Marmary *et al.*, 1987). However, the use of anesthetic and pilocarpine greatly facilitates collection of venom without undue stress to the snake, and total dry yields are still typically much greater than those obtained via other methods such as simple restraint and aspiration.

In conclusion, the administration of ketamine and pilocarpine appears to be tolerated well by several very different species of colubrids, although it should be noted that some species such as *H. gigas* tend to be sensitive to ketamine. The use of this method to obtain sufficient amounts of venom for detailed analyses now appears feasible even for small species, such as *Tantilla nigriceps*, which typically show extremely low venom yields. With the utilization of this technique, in conjunction with sensitive microanalytical techniques, the investigation of colubrid venoms should progress much more rapidly. We are currently investigating biochemical and toxicological properties of the Duvernoy's venoms obtained using this method.

Acknowledgements—Support for this work was provided by grant no. 1-R15-GM52665-01 from the National Institutes of Health to SPM. We thank H. Rosenberg for his helpful comments at an early stage of this work. The following individuals provided snakes or allowed extraction of animals under their care, and their assistance is appreciated: D. Chiszar, J. Hobert, C. Montgomery, W. Sherbrooke, S. S. Sweet and B. Tomberlin. Additional support was provided by a research incentives grant from the University of Northern Colorado Research Corporation and is gratefully acknowledged. Permission to collect several species used in this study was granted by the Colorado Division of Wildlife and the Arizona Game and Fish Department, and their help is also appreciated.

REFERENCES

Bradford, M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analyt. Biochem.* **72,** 248–251.

Chiszar, D., Weinstein, S. A. and Smith, H. M. (1992) Liquid and dry venom yields from brown tree snakes, *Boiga irregularis* (Merrem). In: *Contributions in Herpetology*, pp. 11–14 (Strimple, P. D. and Strimple, J. L., Eds). Cincinnati, OH: Cincinnati Museum of Natural History.

FitzSimons, D. C. and Smith, H. M. (1958) Another rear-fanged South African snake lethal to humans. *Herpetologica* **14,** 198–202.

Gans, C. (1978) Reptilian venoms: some evolutionary considerations. In: *Biology of the Reptilia*, Vol. 8, pp. 1–39 (Gans, C. and Gans, K. A., Eds). New York: Academic Press.

Glenn, J. L., Porras, L. W., Nohavec R. D. and Straight, R. C. (1992) Analysis of the Duvernoy's gland and oral secretions of *Hydrodynastes gigas* (Duméril, Bibron, and Duméril) (Reptilia : Serpentes). In: *Contributions in Herpetology*, pp. 19–26 (Strimple, P. D. and Strimple, J. L., Eds). Cincinnati, OH: Cincinnati Museum of Natural History.

Kornalik, F., Táborská, E. and Mebs, D. (1978) Pharmacological and biochemical properties of a venom gland extract from the snake *Thelotornis kirtlandii*. *Toxicon* **16,** 535–542.

Marmary, Y., Fox, P. C. and Baum, B. J. (1987) Fluid secretion rates from mouse and rat parotid glands are markedly different following pilocarpine stimulation. *Comp. Biochem. Physiol.* **88A,** 307–310.

McAlister, W. H. (1963) Evidence of mild toxicity in the saliva of the hognose snake (*Heterodon*). *Herpetologica* **19,** 132–137.

Minton, S. A. and Weinstein, S. A. (1987) Colubrid snake venoms: immunologic relationships, electrophoretic patterns. *Copeia* **1987,** 993–1000.

Ogawa, H. and Sawai, Y. (1986) Fatal bite of Yamakagashi (*Rhabdophis tigrinus*). *Snake* **18,** 53–54.

Pirkle, H. and Markland, F. S., Eds (1988) *Hemostasis and Animal Venoms*, 628 pp. New York: Marcel Dekker.
Pope, C. H. (1958) Fatal bite of captive African rear-fanged snake (*Dispholidus*). *Copeia* **1958,** 280–282.
Robertson, S. S. D. and Delpierre, G. R. (1969) Studies on African snake venoms—IV. Some enzymatic activities in the venom of the boomslang *Dispholidus typus*. *Toxicon* **7,** 189–194.
Rosenberg, H. I. (1992) An improved method for collecting secretion from Duvernoy's gland of colubrid snakes. *Copeia* **1992,** 244–246.
Rosenberg, H. I., Bdolah, A. and Kochva, E. A. (1985) Lethal factors and enzymes in the secretion from Duvernoy's gland of three colubrid snakes. *J. exp. Biol.* **233,** 5–14.
Shier, W. T. and Mebs, D., Eds (1990) *Handbook of Toxinology*, 814 pp. New York: Marcel Dekker.
Theakston, R. D. G., Reid, H. A. and Romer, J. D. (1979) Biological properties of the red-neck keel back snake (*Rhabdophis subminiatus*). *Toxicon* **17,** Suppl. 1, 190.
Vest, D. K. (1981) The toxic Duvernoy's secretion of the wandering garter snake *Thamnophis elegans vagrans*. *Toxicon* **19,** 831–839.
Vest, D. K. (1988) Some effects and properties of Duvernoy's gland secretion from *Hypsiglena torquata texana* (Texas night snake). *Toxicon* **26,** 417–419.
Vest, D. K., Mackessy, S. P. and Kardong, K. V. (1991) The unique Duvernoy's secretion of the brown tree snake (*Boiga irregularis*). *Toxicon* **29,** 532–535.
Weinstein, S. A. and Kardong, K. V. (1994) Properties of Duvernoy's secretions from opisthoglyphous and aglyphous colubrid snakes. *Toxicon* **32,** 1161–1185.
Weinstein, S. A., Chiszar, D., Bell, R. C. and Smith, L. A. (1991) Lethal potency and fractionation of Duvernoy's secretion from the brown tree snake *Boiga irregularis*. *Toxicon* **29,** 401–408.
Weinstein, S. A., Stiles, B. G., McCoid, M. J., Smith, L. A. and Kardong, K. V. (1993) Variation of lethal potencies and acetylcholine receptor binding activity of Duvernoy's secretions from the brown tree snake *Boiga irregularis* Merrem. *J. nat. Toxins* **2,** 187–198.