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# Pharmacokinetics of the Sri Lankan hump-nosed pit viper (*Hypnale hypnale*) venom following intravenous and intramuscular injections of the venom into rabbits

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ABSTRACT

The knowledge of venom pharmacokinetics is essential to improve the understanding of envenomation pathophysiology. Using a double-sandwich ELISA, this study investigated the pharmacokinetics of the venom of hump-nosed pit viper (*Hypnale hypnale*) following intravenous and intramuscular injections into rabbits. The pharmacokinetics of the venom injected intravenously fitted a three-compartment model. There is a rapid ($t_{1/2\pi}$ = 0.4 h) and a slow ($t_{1/2\alpha}$ = 0.8 h) distribution phase, followed by a long elimination phase ($t_{1/2\beta}$ = 19.3 h) with a systemic clearance of 6.8 mL $h^{-1}$ $kg^{-1}$, consistent with the prolonged abnormal hemostasis reported in *H. hypnale* envenomation. On intramuscular route, multiple peak concentrations observed in the beginning implied a more complex venom absorption and/or distribution pattern. The terminal half-life, volume of distribution by area and systemic clearance of the venom injected intramuscularly were nevertheless not significantly different ($p > 0.05$) from that of the venom injected intravenously. The intramuscular bioavailability was exceptionally low ($F_{i.m.}$ = 4%), accountable for the highly varied median lethal doses between intravenous and intramuscular envenomations in animals. The findings indicate that the intramuscular route of administration does not significantly alter the pharmacokinetics of *H. hypnale* venom although it significantly reduces the systemic bioavailability of the venom.



## 1. Introduction

Snake envenomation is a neglected tropical disease and a disease of poverty (Gutiérrez et al., 2006; Harrison et al., 2009; WHO, 2010). There are approximately 5.5 million snakebite cases yearly, of these at least 4,21,000 envenomings and 20,000 deaths occur worldwide, although the figures in reality may soar as high as 1,841,000 envenomings and 94,000 deaths (Kasturiratne et al., 2008). The problem is common in developing and under-developed countries, affecting mainly agricultural workers who are usually the sole breadwinners in the families.

Optimization of snakebite management and antivenom use rely greatly on the toxinological characterization of a venom. The essential knowledge from which includes not only the venom's composition and toxic activities, but also its disposition in the body i.e. the pharmacological profile. Unfortunately, for over half a century, the management of

*Abbreviations:* anti-*Hh*, anti-*H. hypnale* venom; AUC, area under the curve; $CL_1$, systemic clearance by body weight; ELISA, enzyme-linked immunosorbent assay; h, hour; $H_2SO_4$, sulfuric acid; HRP, horseradish peroxidase; *i.m.*, intramuscular; *i.v.*, intravenous; OPD, ortho-phenylenediamine; PBS, phosphate-buffered saline; $t_{1/2\alpha}$, half-life at distribution phase; $t_{1/2\beta}$, half-life at elimination phase; $V_{D(area)}$, volume of distribution by area.

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snakebite is still confronted with various issues pertaining to antivenom supplies or uses (Williams et al., 2011), and the existing protocols for the therapeutic uses of antivenom are largely empirical (Chippaux, 1998). There is generally a lack of knowledge on the pharmacokinetics of individual snake venom and toxin, especially on the important aspects such as the venom's distribution and elimination half-lives, systemic clearance and bioavailability. These pharmacokinetic processes or parameters temporally govern the serum concentrations of venom, which is known to correlate clinically with the severity of systemic envenomation syndrome (Hung et al., 2003). To date, although there have been some studies on the pharmacokinetics of snake venoms or toxins using animal models (Guo et al., 1993; Audebert et al., 1994; Barral-Netto and von Sohsten, 1991; Ismail et al., 1996, 1998; Nakamura et al., 1995; Pakmanee et al., 1998; Mello et al., 2010; Paniagua et al., 2012; Tseng et al., 1968; Yap et al., 2013), it is well observed that venoms are highly variable from species to species and the pharmacokinetic parameters cannot be overall generalized across all snake venoms. Some detailed pharmacokinetic information such as systemic bioavailability of venom following experimental envenomation is even scarcer in the literature. A good understanding of the venom pharmacokinetics of the venom as a result of envenomation is essential to elucidate the time course of envenomation pathophysiology, thereby improving clinical monitoring and treatment protocol of snake envenoming.

Envenomation by the hump-nosed pit viper, *Hypnale hypnale*, has received much attention in recent years following its re-authentication as the leading cause of snakebites with fatal outcome in Sri Lanka and Western Ghats of India (Joseph et al., 2007; Ariaratnam et al., 2008; Alirol et al., 2010). Envenomation by *H. hypnale* can cause severe complications e.g. local tissue destruction, venom-induced coagulopathy and acute kidney injury; however, there is no specific antivenom clinically available for the treatment. The venom has been studied for its biochemical and toxinological properties (Tan et al., 2011a; Maduwage et al., 2011), as well as cross-neutralization activity by paraspecific antivenoms against the major toxic effects of *H. hypnale* venom (Tan et al., 2011b, 2012a). The knowledge on the venom's pharmacokinetic properties e.g. half-lives, clearance and bioavailability is however lacking. In the present study, we investigated the pharmacokinetics of *H. hypnale* venom following intravenous and intramuscular administrations of the whole venom into rabbits.

## 2. Materials and methods

### 2.1. Venoms

*H. hypnale* venom was a pooled sample obtained from the milking of >10 adult snakes captured in Sri Lanka (Gamapha, Kelaniya, Avissawela, Colombo regions). The snakes were kept at the serpentarium at the University of Colombo, Sri Lanka, and were identified by Anslem de Silva, an expert herpetologist.

### 2.2. Laboratory animals

New Zealand white rabbits were supplied by Laboratory Animal Centre, Faculty of Medicine, University of Malaya. The use of animals was approved by the Institutional Animal Care and Use Committee of the Faculty of Medicine of the University of Malaya [ethics clearance number: PM/03/03/2010/FSY(R)], and the animals were handled in strict accordance with the recommendations in the guidelines given by CIOMS on animal experimentation (Howard-Jones, 1995).

### 2.3. Apparatus and reagents

Ninety-six-well flat bottom microtiter plates (Nunc, Denmark), Goat anti-rabbit IgG-horseradish peroxidase conjugate (HRP) (Bio-Rad Laboratories, USA), PBS-Tween 20 (Sigma, USA), ortho-phenylenediamine (OPD) (Sigma, USA), HiTrap™ Protein A-Sepharose 5-mL column (GE Healthcare, Sweden), Vivaspin® ultrafiltration devices (Sartorius Stedim Biotech, Germany) were purchased from the manufacturers. All other chemicals and reagents utilized were of analytical grade (Sigma Chemical Company or Merck).

### 2.4. Immunization, production of anti-H. hypnale IgG and horseradish peroxidase (HRP) conjugate

The immunization and antibody preparation were achieved as described by Tan et al. (2012b) in developing a double-sandwich ELISA for venom detection and quantitation. In brief, rabbits ($n = 3$) of 2 kg each were initially immunized intramuscularly over the back muscles with *H. hypnale* venom (50 μg in 0.5 mL PBS, pH 7.4) emulsified in 0.5 mL complete Freund's adjuvant, followed by three subsequent intramuscular doses of 100 μg venom in 0.5 mL PBS emulsified in 0.5 mL incomplete Freund's adjuvant at fortnightly intervals. Blood (2 mL) was collected via the rabbits' marginal ear veins, just before each immunization, including the first dose. The blood sample was left to clot in plain tubes at room temperature, and subsequently centrifuged at 3000 *g* to separate the serum. From the serum (antiserum), the antibody titer levels (at two weeks following each injection) were monitored by indirect ELISA.

The immunoglobulin G (IgG) purification from the antiserum was carried out with protein A affinity column as described by Hudson and Hay (1980) with slight modification. Conjugation of IgG with HRP was carried out according to the periodate oxidation method described by Tijssen (1985).

### 2.5. Double-sandwich ELISA for venom detection and quantitation

The methodology was adapted from Tan et al. (2012b). In brief, microtiter wells were each coated with 100 μL of anti-*H. hypnale* (anti-*Hh*) IgG (4 μg $ml^{-1}$) overnight at 4 °C. After washing with 100 μL PBS-Tween 20 for four times, the wells were then incubated for 2 h at room temperature with 100 μL of rabbit sera (1:10) from pharmacokinetic experiments at various time intervals. Following another



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