Pharmacokinetics of *Naja sumatrana* (Equatorial Spitting Cobra) Venom and Its Major Toxins in Experimentally Envenomed Rabbits

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Abstract

**Background:** The optimization of snakebite management and the use of antivenom depend greatly on the knowledge of the venom's composition as well as its pharmacokinetics. To date, however, pharmacokinetic reports on cobra venoms and their toxins are still relatively limited. In the present study, we investigated the pharmacokinetics of *Naja sumatrana* (Equatorial spitting cobra) venom and its major toxins (phospholipase A₂, neurotoxin and cardiotoxin), following intravenous and intramuscular administration into rabbits.

**Principal findings:** The serum antigen concentration-time profile of the *N. sumatrana* venom and its major toxins injected intravenously fitted a two-compartment model of pharmacokinetics. The systemic clearance (91.3 ml/h), terminal phase half-life (13.6 h) and systemic bioavailability (41.9%) of *N. sumatrana* venom injected intramuscularly were similar to those of *N. sputatrix* venom determined in an earlier study. The venom neurotoxin and cardiotoxin reached their peak concentrations within 30 min following intramuscular injection, relatively faster than the phospholipase A₂ and whole venom (Tₘₐₓ = 2 h and 1 h, respectively). Rapid absorption of the neurotoxin and cardiotoxin from the injection site into systemic circulation indicates fast onsets of action of these principal toxins that are responsible for the early systemic manifestation of envenoming. The more prominent role of the neurotoxin in *N. sumatrana* systemic envenoming is further supported by its significantly higher intramuscular bioavailability (Fₘᵢᵢ = 81.5%) compared to that of the phospholipase A₂ (Fₘᵢᵢ = 68.6%) or cardiotoxin (Fₘᵢᵢ = 45.6%). The incomplete absorption of the phospholipase A₂ and cardiotoxin may infer the toxins’ affinities for tissues at the injection site and their pathological roles in local tissue damages through synergistic interactions.

**Conclusion/Significance:** Our results suggest that the venom neurotoxin is absorbed very rapidly and has the highest bioavailability following intramuscular injection, supporting its role as the principal toxin in systemic envenoming.

**Citation:** Yap MKK, Tan NH, Sim SM, Fung SY, Tan CH (2014) Pharmacokinetics of *Naja sumatrana* (Equatorial Spitting Cobra) Venom and Its Major Toxins in Experimentally Envenomed Rabbits. PLoS Negl Trop Dis 8(6): e2890. doi:10.1371/journal.pntd.0002890

**Editor:** José María Gutiérrez, Universidad de Costa Rica, Costa Rica

**Received January 17, 2014; Accepted April 8, 2014; Published June 5, 2014**

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**Funding:** This work was supported by PV 054/2011B and High Impact Research Grant UM.C/625/1/HIR/ /E20040-20001 from the University of Malaya, Kuala Lumpur, Malaysia. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

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Introduction

Snake envenomation remains a neglected tropical disease prevalent in the Southeast Asia region, including Malaysia [1,2]. It affects not only the population in the rural area but also the suburban regions due to rapid urbanization, and the encroaching of human activities into the natural habitat of snakes [3–7]. In Malaysia, cobra bites appears to be one of the commonest causes of snake envenomation [4–6]. There are two species of common cobras in Malaysia: *Naja kaouthia* and *Naja sumatrana*, both classified as Category 1 medically important venomous snake [2]. Of these two *Naja* cobras, *N. sumatrana* is widely distributed in the Peninsula Malaysia (including Singapore), and is also known as the Equatorial spitting cobra [8], one of the venom-spitting species in Southeast Asia that are able to cause venom ophthalmia. Clinically, cobra bites produce systemic envenomation syndrome with the characteristic neuromuscular paralysis, and local toxicity manifested as severe tissue necrosis [2,6,9]. The characterization of different cobra venoms, however, are necessary for the better understanding of cobra envenomation pathophysiology as the toxin compositions in cobra venoms vary from species to species [10]. Recent venom profiling with the use of ion-exchange high performance liquid chromatography has shown that the major toxins of *N. sumatrana* venom comprise high abundance of phospholipase A₂ and three-finger toxins such as polypeptides of neurotoxins and cardiotoxins [10]. These are toxins with varied biological and physicochemical properties which make the characterizations of individual toxins warranted in order to gain better insights into the toxic effects of the whole venom. The optimization of snakebite management and the use of antivenom depend greatly on the knowledge of the venom’s composition, pharmacological activities, as well as its disposition in the body (i.e. pharmacokinetics). The pathophysiological and pharmacological effects of snake envenomation are related to the absorption and
distribution kinetics of venom toxins into the systemic circulation. Indeed, it has been reported that the serum concentrations of venom antigens in snakebite victims are well correlated with the severity of systemic and local symptoms during the course of envenomation [11]. Although there have been some studies on the pharmacokinetics of snake venoms or toxins in animals [12–22], the highly varied snake venom compositions, inconsistent animal models, different pharmacokinetic modelling make the convergence of the data equivocal to have the pharmacokinetic parameters generalized across all snake species. To date, even within the Naja genus of cobras, the pharmacokinetic studies on their venoms were limited to isolated toxins of Formosan cobra [12,21], a few African cobra venoms and their alpha toxins [15] and N. sputatrix venom [22]. Information on the systemic bioavailability of cobra venoms and their toxins following envenomation is even scarcer in the literature. There is therefore a need to define the pharmacokinetic parameters of specific cobra venom and its toxins more meticulously for better clinical correlation.

In the present study, the pharmacokinetics of N. sumatrana venom and its three major types of toxins (neurotoxin, cardiotoxin and phospholipase A$_2$) were investigated using double-sandwich ELISA. This is the first report where the pharmacokinetics of a cobra venom was investigated alongside the pharmacokinetics of all its major types of toxins. The results will make it possible to interpret the pharmacokinetics of the whole venom in the light of that of its major toxins, and to enable better understanding of the pathophysiological effects of the venom.

**Methods**

**Ethical statements**

All experimental animals were handled in accordance to CIOMS guidelines on animal experimentation [23]. The experimental protocol on the animal study (2013-06-07/MOL/R/FSY) was approved by the Institutional Animal Care and Use Committee, Faculty of Medicine, University of Malaya.

**Venom, reagents and separation media**

The venom was a pooled sample obtained from three adult N. sumatrana captured in central Malaysia (Negeri Sembilan) and was supplied by Snake Valley (Seremban, Malaysia).

Resource S ion exchange column and HiTrap Protein A HP affinity column were purchased from GE Healthcare (New Jersey, USA). Goat anti-rabbit IgG-horseradish peroxidase (HRP) conjugate was obtained from Abnova, Taipei, Taiwan. IChrosphere WP 300 C$_{18}$ reverse-phase column cartridge was purchased from Merck, New Jersey, USA. iBlot Gel Transfer stacks and iBlot blotting system were supplied by Invitrogen. Sephaxex G-25 gel beads and all other reagents were purchased from Sigma – Aldrich (St. Louis, USA) or as stated in the methods.

**Animals**

The animals used in this study (New Zealand white rabbits) were supplied by Chenur Supplier (Selangor, Malaysia). The animals were housed in Laboratory Animal Centre, Faculty of Medicine, University of Malaya, and received water and food ad libitum.

**Purification of N. sumatrana toxins**

The major N. sumatrana venom toxins (phospholipase A$_2$, neurotoxin and cardiotoxin) were isolated from the venom by Resource S ion-exchange chromatography as described by Yap et al., 2011 [10]. The isolated phospholipase A$_2$, neurotoxin and cardiotoxin (corresponds to peak 5, peak 7 and peak 8, respectively as reported in Yap et al., 2011 [10]) were further purified by C$_{18}$ reverse-phase high-performance liquid chromatography (HPLC) to homogeneity on 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel bands were subjected to in-gel tryptic digestion followed by protein identification using matrix assisted laser desorption/ionization-time of flight (MALDI-TOF/TOF) mass spectrometry, as described by Yap et al., 2011 [10].

**Production of IgG antibodies against N. sumatrana venom, venom phospholipase A$_2$, neurotoxin and cardiotoxin**

Pre-immune serum was collected and used as the control in ELISA. In the first immunization, N. sumatrana venom (10 µg) or venom toxins (neurotoxin, cardiotoxin and phospholipase A$_2$, respectively at 5 µg) dissolved in PBS (pH 7.2) and mixed with an equal volume of Freund’s complete adjuvant, was injected intramuscularly into the thigh of the rabbits (n = 3 for each group). For the subsequent immunizations, 20 µg of the venom or 10 µg of venom toxins were dissolved in PBS (pH 7.2), respectively, mixed with an equal volume of Freund’s incomplete adjuvant and injected intramuscularly at multiple sites at the back of the rabbit fortnightly for 8 weeks. The immunogenicity and antibody titers of inocula were monitored using indirect ELISA as described by Yap et al., 2011 [10]. The rabbits were bled by cardiac puncture 9 days after the final immunization as indicated by plateauing of antibody titer on indirect ELISA.

**Purification of IgG and preparation of horseradish peroxidase (HRP) conjugate**

Anti - N. sumatrana venom IgG and three anti-toxins IgG were isolated from rabbit sera (upon completion of immunization scheme) by Sephadex G-25 gel chromatography, followed by Protein A affinity chromatography [24]. The IgG-HRP conjugate was prepared as described by Wisdom, 1996 [25].

**Investigation of immunological cross-reactivity of N. sumatrana major venom toxins (phospholipase A$_2$, PLA$_2$; neurotoxin, NTX; cardiotoxin, CTX)**

**Indirect ELISA for investigation of immunological cross-reactivity.** ELISA immunoplate was coated with 100 ng/ml of venom toxin (phospholipase A$_2$, PLA$_2$; neurotoxin, NTX or cardiotoxin, CTX) respectively and incubated overnight at 4°C. The plates were washed with PBS-Tween and subsequently anti-PLA$_2$ IgG, anti-NTX IgG or anti-CTX IgG (dilutions 1:200) was added and allowed to incubate at room temperature for 1 h. This
was followed by incubation with goat anti-rabbit IgG horseradish peroxidase conjugate (dilutions of 1:6000) and 100 μl of substrate o-phenylenediamine dihydrochloride (0.4 mg/ml) for 1 h. The reaction was terminated by adding 50 μl sulfuric acid (12.5%). The absorbance at 492 nm was then determined using Bio-Rad Model 690 microplate reader. The degree of cross-reactivity was expressed in percentage (%) of absorbance.

**Double-sandwich ELISA for investigation of immunological cross-reactivity.** ELISA immunoplate was coated overnight at 4°C with 100 μl of anti-PLA2 IgG, anti-NTX IgG, anti-CTX IgG (4 μg/ml, respectively). Plates were then incubated with 100 μl of the respective venom toxins as antigens (phospholipase A2, neurotoxin, cardiotoxin) at a concentration of 100 ng/ml. This was followed by incubation with 100 μl of anti-toxins IgG-HRP conjugate (1:100) for 2 h, and 100 μl of substrate o-phenylenediamine dihydrochloride (0.4 mg/ml) was then added. The reaction was terminated after 1 h by adding 50 μl of 12.5% sulfuric acid and the absorbance at 492 nm were then determined using Bio-Rad Model 690 microplate reader. The degree of cross-reactivity was expressed in percentage (%) of absorbance.

**SDS-PAGE and western blotting (immunoblotting).** SDS-PAGE was conducted in an electrophoresis (slab) system according to the method described by Studier, 1973 [26] and a Fermentas Spectra Multicolor Broad Range Protein Ladder broad range SDS-PAGE standard was used for calibration. Ten micrograms of venom toxins (phospholipase A2, neurotoxin or cardiotoxin) was electro-phoresed (15% gel) under reducing condition.

The proteins on the polyacrylamide gel was transferred to a polyvinylidene fluoride (PVDF) membrane (iBlot Gel Transfer Stacks, PVDF, mini, Invitrogen) using iBlot blotting system (Invitrogen). The PVDF membrane was subsequently blocked with 2% BSA in Tris-buffered saline, Tween 20. Anti-PLA2 IgG, anti-NTX IgG or anti-CTX IgG (dilution of 1:500 in TBS-Tween) was added to the PVDF membrane followed by incubation with Goat anti-rabbit IgG horseradish peroxidase conjugate (dilutions of 1:1000) for 1 h. The chromogenic detection of the protein bands on the PVDF membrane was carried out by addition of the substrate solution (Novex HRP Chromogenic Substrate (TMB), Invitrogen).

**Determination of serum venom antigen and venom toxin antigen levels using double-sandwich ELISA**

Double-sandwich ELISA was conducted as described previously [27]. It was used to monitor the serum venom antigen levels following experimental envenomation in rabbits (n = 3) during pharmacokinetic studies. Briefly, ELISA immunoplates were coated overnight at 4°C with optimal coating concentration for venom and venom toxins, which has been optimized as stated above in the previous section (double-sandwich ELISA for investigation of immunological cross-reactivity). This was followed by subsequent incubation with 100 μl of diluted rabbit serum samples (1:20) collected at different time intervals, 100 μl of anti-N. sumatrana venom IgG-HRP conjugate and anti-toxins IgG-HRP conjugate (dilution of 1:1000) for 2 h. Substrate o-phenylenediamine dihydrochloride (0.4 mg/ml) was added for colorimetric development and the absorbance at 492 nm was then determined using Bio-Rad Model 690 microplate reader. A standard curve was constructed using varying dilutions of venom or the respective toxins in the spiked pre-envenomed sera.

**Pharmacokinetics of N. sumatrana venom or toxins after intravenous (i.v.) and intramuscular (i.m.) administrations**

The pharmacokinetics of N. sumatrana venom or toxins was studied using rabbits (n = 3). A sub-lethal dose of the venom or toxins was administered intravenously (i.e., marginal ear vein) or intramuscularly (i.m., quadriceps) into rabbits. Doses administered were as follow: venom 0.5 mg/kg (i.m.) or 0.1 mg/kg (i.v.); phospholipase A2 0.1 mg/kg (i.m.) or 0.05 mg/kg (i.v.); neurotoxin 0.07 mg/kg (i.m.) or 0.05 mg/kg (i.v.); cardiotoxin 0.15 mg/kg (i.m.) or 0.05 mg/kg (i.v.). Blood samples were collected from the central ear artery before experimental envenomation and at specific time points (5 min, 10 min, 30 min, 1 h, 2 h, 3 h, 6 h and 24 h) after venom injection. The collected blood samples were centrifuged at 3,500 g for 20 min to obtain the sera, which were kept at -20°C until further analysis. The serum antigen concentrations were measured by double-sandwich ELISA as described above using the pre-envenomed serum sample taken from the same animal as the control for baseline reading.

A parallel series of experiments were conducted to investigate the pharmacokinetics of cardiotoxin in the whole venom when N. sumatrana venom was injected intravenously or intramuscularly into the rabbits (n = 3). The serum concentrations of cardiotoxin (in the whole venom) at specified sampling times were estimated using anti-CTX IgG on a double-sandwich ELISA, as described above. The equivalent amounts of cardiotoxin in the injected whole venom (0.1 mg/kg, i.v. or 0.5 mg/kg, i.m.) were estimated to be 0.04 mg/kg or 0.2 mg/kg, respectively, based on a 40% (by dry mass) composition of the whole venom [10]. This additional study aimed to verify if the pharmacokinetics of cardiotoxin when injected alone would be significantly different from that when injected in its native environment (the whole venom).

**Pharmacokinetic analysis**

The pharmacokinetic parameters of N. sumatrana venom and venom toxins were determined using the method of feathering [28]. The initial phase rate constant (α) and terminal phase rate constant (β) were determined from the slopes of the best-fit lines obtained for the initial phase and terminal phase, respectively, of the log plasma concentration versus time plot. The initial phase half-life (T1/2α) and terminal phase half-life (T1/2 β) were determined by formula T1/2α or T1/2β = 0.693/α or β. The area under the curve (AUC) was calculated from zero time to the last experimental time point by trapezoidal rule and extrapolated to infinity (AUC∞) according to the formula: AUC∞= AUCt + C/β, where t is the last experimental time point and Ct represents the last serum venom concentration determined at time t.

The other important pharmacokinetic parameters were determined as follows:

Systemic clearance, CL = dose (F)/AUC∞ Volume of distribution by area, Vd,area = CL/β Volume of central compartment, Vc = Dose/A (A + B) Volume of peripheral compartment, Vp = k12/k21 (Vc) Fν,nu is the intravenous bioavailability which is 1.

Fν,i.m is the intramuscular bioavailability, and was calculated as follows:

\[ F_{i.m.} = \frac{AUC_{i.m}}{AUC_{i.v.}} \times \frac{Dose_{i.m.}}{Dose_{i.v.}} \]

**Statistical analysis**

All data are reported as the mean ± S.D. or mean (95% C.I.). Mann-Whitney U test was used to compare differences between
two independent groups. Kruskal-Wallis H Test is the nonparametric test for the comparison of more than two independent groups. The level of significance was set at \( p < 0.05 \). The statistical analysis was conducted using SPSS 21.0 (SPSS Inc., Chicago, IL, USA).

**Results**

**Pharmacokinetics of N. sumatrana venom following intravenous administration**

The serum concentration-time profiles of whole *N. sumatrana* venom antigen following a single *i.v.* and *i.m.* administrations of venom into rabbits (\( n = 3 \)) are shown in Figure 1.

The *i.v.* serum concentration-time profile of *N. sumatrana* venom (0.1 mg/kg) (Figure 1, dotted line) showed a bi-exponential pattern which was best fitted to a two-compartment model of pharmacokinetics described by the equation \( C_t = A e^{-\alpha t} + B e^{-\beta t} \); where \( C_t \) represents the concentration at time, \( t \); \( A \) and \( B \) represent the venom concentrations at the zero time intercepts of the initial fast phase and terminal slow phase, respectively; while \( \alpha \) and \( \beta \) represent the first-order disposition rate constants for the initial fast phase and the terminal phase, respectively.

The venom antigen level declined rapidly within the first 1 h (\( T_{1/2a} = 0.8 \pm 0.3 \) h) during the initial phase followed by a much slower decline at the terminal phase (\( T_{1/2b} = 13.6\pm 1.1 \) h). The volume of distribution by area (\( V_{d,area} \)) of the venom antigens in rabbits was \( 1.8 \pm 0.03 \) L, and the systemic clearance (CL) was \( 91.3 \pm 7.8 \) ml/h, and the AUC\( _{0-a} \) was \( 2201.2 \pm 185.5 \) ng/ml.h. The distribution rate constant for transfer from central to peripheral compartment (\( k_{12} = 0.4 \pm 0.2 \) h\(^{-1} \)) was comparable to that from peripheral to central compartment (\( k_{21} = 0.5 \pm 0.2 \) h\(^{-1} \); \( p > 0.05 \)). Consequently, the volume of peripheral compartment (0.8\( \pm 0.2 \) L) calculated based on the ratio of \( k \) constants was comparable to that of central compartment (1.0\( \pm 0.1 \) L).

**Pharmacokinetics of N. sumatrana venom following intramuscular administration**

The intramuscular administration of whole *N. sumatrana* venom in rabbits yielded a serum concentration-time profile (Figure 1, solid line) with the absorption and distribution phase appeared indistinguishable. The venom antigen level peaked within 1 h at a concentration (\( C_{max} \)) of 391.7\( \pm 48.5 \) ng/ml. The terminal half-life (\( T_{1/2b} = 12.5\pm 0.9 \) h), volume of distribution by area (\( V_{d,area} = 1.7 \pm 0.1 \) L) and the systemic clearance (CL = 94.8\( \pm 12.7 \) ml/h) of the venom antigen following *i.m.* injection were not significantly different from that of *i.v.* pharmacokinetic parameters (\( p > 0.05 \)) (Table 1).

The AUC\( _{0-a} \) of *N. sumatrana* venom when injected intramuscularly (0.5 mg/kg) was \( 4617.8 \pm 583.8 \) ng/ml.h. However, when adjusted to the intravenous venom dose (0.1 mg/kg), the normalized AUC\( _{0-a} \) of the venom antigens following *i.m.* administration was \( 923.6 \pm 116.8 \) ng/ml.h, which was significantly lower than the *i.v.* AUC\( _{0-a} \) value (2201.2\( \pm 185.5 \) ng/ml.h; \( p < 0.05 \)). The *i.m.* bioavailability (\( F_{i.m.} \)) calculated from the two AUC\( _{0-a} \) values were 41.9\( \pm 0.2 \)%.

**Immunological cross-reactions of N. sumatrana venom toxins (phospholipase A\(_2\), neurotoxin and cardiotoxin)**

The phospholipase A\(_2\), neurotoxin and cardiotoxin were isolated and purified from *N. sumatrana* venom. The protein identity of each toxin was confirmed by MALDI-TOF/TOF and is shown in Table 2.

Indirect ELISA and double-sandwich ELISA demonstrated extensive cross-reactions between phospholipase A\(_2\) and neuro-
Pharmacokinetics of Cobra Venom Toxins

Table 1. Pharmacokinetic parameters of *Naja sumatrana* venom following intravenous and intramuscular administrations of the venom into rabbits.

| Parameters | Intravenous (i.v.) (LD$_{50}$ = 0.5 µg/g) | Intramuscular (i.m.) (LD$_{50}$ = 0.8 µg/g) |
|------------|------------------------------------------|------------------------------------------|
| A (ng/ml)  | 101.0 ± 17.4                             | -                                        |
| $\alpha$ (h$^{-1}$) | 0.9 ± 0.4                                 | -                                        |
| B (ng/ml)  | 100.3 ± 1.4                               | 247.7 ± 33.1                             |
| $\beta$ (h$^{-1}$) | 0.05 ± 0.0004                             | 0.06 ± 0.004                             |
| $T_{1/2\ a}$ (h) | 0.8 ± 0.3                                 | -                                        |
| $T_{1/2\ b}$ (h) | 13.6 ± 1.1                                | 12.5 ± 0.9                               |
| $C_{max}$ (ng/ml) | -                                       | 391.7 ± 48.5                             |
| $k_{12}$ (h$^{-1}$) | 0.4 ± 0.2                                 | -                                        |
| $k_{21}$ (h$^{-1}$) | 0.5 ± 0.2                                 | -                                        |
| $V_{darea}$ (L) | 1.8 ± 0.03                               | 1.7 ± 0.1                                |
| $V_{p}$ (L) | 1.0 ± 0.1                                 | -                                        |
| $k_{p}$ (h$^{-1}$) | 0.8 ± 0.2                                 | -                                        |
| CL (ml/h)  | 91.3 ± 7.8                                | 94.8 ± 12.7                              |
| AUC$_{0-\infty}$ (ng/ml.h) | 2201.2 ± 185.5             | 4617.8 ± 583.8                           |
| Bioavailability, F (%) | 100 (by definition) | 41.9 ± 0.2                              |

The dose of *N. sumatrana* venom injected into rabbits (n = 3) were i.v.: 0.1 mg/kg and i.m.: 0.5 mg/kg. Data were expressed as mean ± S.D. for n = 3.

* AUC$_{0-\infty}$ value was adjusted to the dosage of i.v. injection, i.e. 0.1 mg/kg.

doi:10.1371/journal.pntd.0002890.t001

Table 2. MALDI-TOF/TOF identification of phospholipase A$_2$, neurotoxin and cardiotoxin isolated from *Naja sumatrana* venom.

| Venom toxins | Matched peptide sequences | Accession No. and Protein Family | Protein Score | % coverage |
|--------------|---------------------------|---------------------------------|---------------|------------|
| Phospholipase A$_2$ | SWWHFADYGCAVCGR | Q92084 Neutral Phospholipase A$_2$- A | 527 | 51 |
|               | GGSFTPVDOPE |                          |               |            |
|               | CCQHDNCYNEAEK |                          |               |            |
|               | CWPYFK |                          |               |            |
|               | TSYECSQGLTCK |                          |               |            |
|               | GGNNAACAAVCDCCDR |                          |               |            |
| Neurotoxin | LECHDQSOSSPTTGTGCGG | Q995N6 Short neurotoxin | 82 | 74 |
|             | ETNCYK |                          |               |            |
|             | NGIEINCCDDAR |                          |               |            |
| Cardiotoxin | LVPYFYK | P60302 Cardiotoxin 3 | 233 | 39 |
|             | MFMVATPK |                          |               |            |
|             | RGCIVPCP |                          |               |            |
|             | GCIIDCPCP |                          |               |            |
|             | YVCCNTDR |                          |               |            |

Protein scores greater than 67 are significant (p<0.05). The mass spectra acquired were searched against all non-redundant NCBI protein database with taxonomy set to Serpentes (taxid: 8570).

doi:10.1371/journal.pntd.0002890.t002
Table 3. Immunological cross-reactivity of *Naja sumatrana* venom toxins by indirect ELISA and double-sandwich ELISA.

| Venom Toxins | % cross-reactivity | Venom Toxins | % cross-reactivity |
|--------------|--------------------|--------------|--------------------|
| anti-PLA2 IgG | 100                | anti-PLA2 IgG | 100                |
| PLA2         | 100                | PLA2         | 100                |
| NTX          | 155.9±26.7         | NTX          | 59.4±13.4          |
| CTX          | 0.8±0.3            | CTX          | 0.7±0.3            |
| anti-NTX IgG | 55.1±2.2           | anti-NTX IgG | 72.5±10.1          |
| PLA2         | 100                | PLA2         | 100                |
| NTX          | 100                | NTX          | 100                |
| CTX          | 0.8±0.2            | CTX          | 0.7±0.3            |
| anti-CTX IgG | 3.2±0.4            | anti-CTX IgG | 0                  |
| PLA2         | 0                  | PLA2         | 0                  |
| NTX          | 0                  | NTX          | 0                  |
| CTX          | 100                | CTX          | 100                |

For indirect ELISA, the immunoplate was coated with 100 ng/ml of the venom toxin as antigen, and reacted with anti-PLA2 IgG, anti-NTX IgG and anti-CTX IgG (dilution of 1:200), respectively. For double-sandwich ELISA, the immunoplate was coated with 4 μg/ml of anti-PLA2 IgG, anti-NTX IgG and anti-CTX IgG respectively; and subsequently incubated with 100 ng/ml of venom toxin.

The venom toxins used were phospholipase A2 (PLA2), neurotoxin (NTX) and cardiotoxin (CTX). Data were expressed as mean ± S.D. for *n* = 3.

doi:10.1371/journal.pntd.0002890.t003

Figure 2. Immunological cross reactions between *N. sumatrana* venom toxins as analyzed by immunoblotting. Venom toxins (10 μg each of phospholipase A2, neurotoxin and cardiotoxin) was electrophoresed on a SDS-PAGE gel (15%, reducing condition), and electro-transferred to a PVDF membrane. This was followed by subsequent incubation with primary antibody (anti-PLA2 IgG, anti-NTX IgG and anti-CTX IgG (dilution of 1:500) and goat anti-rabbit IgG-HRP (dilution of 1:1000). Substrate solution (Novex HRP Chromogenic Substrate (TMB), Invitrogen) was added for colorimetric development.

doi:10.1371/journal.pntd.0002890.g002
Trp or Met residues in the neurotoxin [29]. Similar observation of abnormally high molecular mass neurotoxin has also been reported from Ophiophagus hannah venom [30].

Pharmacokinetics of major toxins following intramuscular and intravenous administration

The serum concentration-time profiles of purified N. sumatrana venom phospholipase A₂, neurotoxin and cardiotoxin following single i.v. or i.m. administrations into rabbits (n = 3) are shown in Figure 3A–C. All of the intravenous profiles showed a bi-exponential pattern which was best fitted to a two-compartment pharmacokinetic model represented by the following equation:

\[ C_t = A e^{-a_t} + B e^{-b_t} \]

where \( C_t \) is the antigen concentration at time \( t \), \( A \) and \( B \) are the areas under the curve for the fast and slow phases, respectively, and \( a_t \) and \( b_t \) are the respective rate constants of elimination. The antigen concentrations in general decreased rapidly within a distribution half-life (\( T_{1/2a} \)) of 0.5–0.7 h during the initial phase and followed by a declining terminal phase with half-life (\( T_{1/2b} \)) of 8–12 h. On intramuscular routes, it contrast to the multiple peaks (\( C_{max} \)) in the case of whole venom, we observed a single peak for toxin absorption at 0.5 h (\( T_{max} \) for neurotoxin and cardiotoxin) or 2 h (\( T_{max} \) for phospholipase A₂) (Figure 3A–C, solid line). The intramuscular profile subsequently followed that of intravenous profile with a linear declining curve, illustrating the terminal phase of the serum concentration-time course.

The i.v. and i.m. pharmacokinetic parameters of all three major toxins were shown in Table 4. Most of the key i.m. pharmacokinetic parameters of the toxins (especially related to distribution and elimination processes) were not significantly different from the corresponding i.v. pharmacokinetic parameters (\( p > 0.05 \)). The intramuscular bioavailability (\( F_{i.m.} \)) of the toxins were estimated by comparing the dose-adjusted intramuscular AUC\(_{0-\infty} \) of toxin to the corresponding intravenous AUC\(_{0-\infty} \).

Pharmacokinetics of cardiotoxin following intravenous or intramuscular administrations of whole N. sumatrana venom

The dotted-line curve in Figure 3D shows the serum cardiotoxin antigen following intravenous whole venom administration that declined in a bi-exponential manner; while the solid-line curve depicts its intramuscular absorption with a

Figure 3. Serum concentration-time profiles of N. sumatrana venom toxins. The serum toxin-antigen concentrations of each individual toxin following intravenous (i.v.) (dotted line) and intramuscular (i.m.) (solid line) injection of N. sumatrana venom phospholipase A₂ (3A), neurotoxin (3B) and cardiotoxin (3C). Figure 3D shows the serum cardiotoxin antigen concentrations following injection of the whole N. sumatrana venom. Toxin concentrations were determined by respective double-sandwich ELISA. Data are given as means \( \pm \) S.D. (n = 3). The insert shows serum concentration-time profile (in arithmetic plot) during the first 3 h.

doi:10.1371/journal.pntd.0002890.g003
Table 4. Pharmacokinetic parameters of *Naja sumatrana* venom toxins (phospholipase A₂, neurotoxin and cardiotoxin) following intravenous and intramuscular administration of the venom toxins into rabbits.

| Venom toxins     | Phospholipase A₂ | Neurotoxin | Cardiotoxin |
|------------------|------------------|------------|-------------|
|                  | Intravenous (i.v. 0.05 mg/kg) | Intramuscular (i.m. 0.07 mg/kg) | Intravenous (i.v. 0.05 mg/kg) | Intramuscular (i.m. 0.015 mg/kg) |
| A (ng/ml)        | 141.7 ± 9.6      | 67.5 ± 6.2 | 63.6 ± 4.2  | -             |
| t (h⁻¹)          | 1.1 ± 0.1        | 1.4 ± 0.3 | 1.3 ± 0.2   | -             |
| B (ng/ml)        | 52.6 ± 3.9       | 44.0 ± 5.4 | 41.4 ± 1.3  | 59.9 ± 2.2    |
| β (h⁻¹)          | 0.06 ± 0.004     | 0.08 ± 0.008 | 0.08 ± 0.005 | 0.08 ± 0.001  |
| T₁/₂ (h)         | 0.7 ± 0.03       | 0.5 ± 0.1 | 0.6 ± 0.1   | -             |
| T₁/₂ (h)         | 11.7 ± 0.8       | 10.18 ± 1.18 | 8.6 ± 0.5  | 8.6 ± 0.1     |
| Cmax (ng/ml)     | 226.6 ± 5.5      | 226.0 ± 2.4 | 120.7 ± 5.5 | 133.0 ± 5.7   |
| k₁₂ (h⁻¹)        | 0.6 ± 0.02       | 0.7 ± 0.1 | 0.6 ± 0.1   | -             |
| k₂₁ (h⁻¹)        | 0.3 ± 0.04       | 0.6 ± 0.1 | 0.6 ± 0.1   | -             |
| V₁₂ (L)          | 1.6 ± 0.03       | 2.1 ± 0.3 | 2.2 ± 0.2   | 2.0 ± 0.03    |
| Vᵢ (L)           | 0.5 ± 0.02       | 0.9 ± 0.1 | 1.0 ± 0.1   | -             |
| Vᵢ (L)           | 0.9 ± 0.1        | 1.0 ± 0.1 | 1.1 ± 0.02  | -             |
| CL (ml/h)        | 95.8 ± 4.8       | 164.1 ± 16.1 | 173.7 ± 2.1 | 173.7 ± 0.001 |
| AUC₀-₆ (ng/ml.h) | 1045.4 ± 52.6    | 611.4 ± 43.3 | 575.7 ± 7.0 | 788.3 ± 23.9  |
| promoted (L)     | (750.0 ± 77.0)*  | (496.2 ± 1.7)* | (262.7 ± 8.0)* | -             |

Bioavailability, F (%) = 100

Data were expressed as mean ± S.D. for n = 3. * AUC₀-₆ values were adjusted to the dosage of i.v. injection, i.e. 0.05 mg/kg for phospholipase A₂, neurotoxin and cardiotoxin.

doi:10.1371/journal.pntd.0002890.t004

Table 5. Pharmacokinetics parameters of cardiotoxin following intravenous and intramuscular administration of whole *Naja sumatrana* venom into rabbits.

| Parameters                  | Intravenous Injection of whole venom | Intravenous Injection of cardiotoxin | Intramuscular Injection of whole venom | Intramuscular Injection of cardiotoxin |
|-----------------------------|-------------------------------------|--------------------------------------|----------------------------------------|----------------------------------------|
| A (ng/ml)                   | 61.3 ± 1.2                          | 63.6 ± 4.2                           | -                                      | -                                      |
| t (h⁻¹)                     | 1.4 ± 0.03                          | 1.3 ± 0.2                            | -                                      | -                                      |
| B (ng/ml)                   | 39.1 ± 1.2                          | 41.4 ± 1.3                           | 73.7 ± 3.0                             | 59.9 ± 2.2                             |
| β (h⁻¹)                     | 0.06 ± 0.001                        | 0.08 ± 0.001                         | 0.1 ± 0.004                            | 0.09 ± 0.001                           |
| T₁/₂ (h)                    | 0.5 ± 0.01                          | 0.6 ± 0.1                            | -                                      | -                                      |
| T₁/₂ (h)                    | 11.0 ± 0.2                          | 8.6 ± 0.1                            | 11.6 ± 0.9                             | 8.2 ± 0.1                              |
| Cmax (ng/ml)                | 169.4 ± 8.5                         | 133.0 ± 5.7                          | -                                      | -                                      |
| k₁₂ (h⁻¹)                   | 0.8 ± 0.01                          | 0.6 ± 0.1                            | -                                      | -                                      |
| k₂₁ (h⁻¹)                   | 0.6 ± 0.02                          | 0.5 ± 0.1                            | -                                      | -                                      |
| V₁₂ (L)                     | 1.9 ± 0.1                           | 2.2 ± 0.1                            | 2.0 ± 0.2                              | 2.0 ± 0.03                             |
| Vᵢ (L)                      | 0.8 ± 0.01                          | 1.0 ± 0.1                            | -                                      | -                                      |
| Vᵢ (L)                      | 1.0 ± 0.04                          | 1.1 ± 0.02                           | -                                      | -                                      |
| CL (ml/h)                   | 119.8 ± 2.4                         | 173.7 ± 2.1                          | 121.7 ± 0.2                            | 173.7 ± 0.001                          |
| AUC₀-₆ (ng/ml.h)            | 668.0 ± 13.0                        | 575.7 ± 7.0                          | 1320.1 ± 35.6                          | 788.3 ± 23.9                           |
| promoted (L)                | (264.0 ± 7.1)*                      | (262.7 ± 4.0)*                       | (262.7 ± 8.0)*                         | -                                      |

Bioavailability, F (%) = 100

The sub-lethal dose of *N. sumatrana* venom injected into rabbits (n = 3, approximately 2 kg each) were i.v.: 0.1 mg/kg and i.m.: 0.5 mg/kg.

Data were expressed as mean ± S.D. for n = 3. The pharmacokinetic parameters when only cardiotoxin was injected are taken from Table 4.

* AUC₀-₆ values were adjusted to the dosage of i.v. injection, i.e. 0.1 mg/kg.

doi:10.1371/journal.pntd.0002890.t005
T_{max} of 0.5 h and a terminal phase parallel to that of intravenous profile. The pharmacokinetic parameters of the “in-venom” cardiotoxin following the intravenous and intramuscular administrations are shown in Table 5. The pharmacokinetic parameters of cardiotoxin when only the toxin was administered are also listed for comparison (see Discussion). Most of the pharmacokinetic parameters of the in-venom cardiotoxin were comparable with values obtained when only purified cardiotoxin was administered, with the major exceptions of a longer elimination half-life (T_{1/2b}) and a lower clearance (CL) for the in-venom cardiotoxin (Table 5).

Discussion

Generally, the serum concentration-time profile of the venom/toxin injected intravenously can be described by an open two-compartment pharmacokinetic model where the venom or toxin is distributed between the central and peripheral compartments. The distribution half-life of N. sumatrana venom (T_{1/2b} = 0.77 h) is comparable to the value obtained for N. sputatrix venom in an earlier study (T_{1/2b} = 0.5 h) [22], and to that for the African cobra venoms (T_{1/2b} = 22.2–30.5 min) [15] although a three-compartment pharmacokinetic model was applied in the latter case. The terminal half-life (T_{1/2b} = 13.6 h) of N. sumatrana venom was not significantly different to that of N. sputatrix venom (T_{1/2b} = 15.4 h) [22], indicating that the elimination of the venom antigens of these two Southeast Asian spitting cobras occurred at similar rate.

The volume of central compartment approximated 1 L for N. sumatrana venom, indicating that on intravenous administration, the venom distributes rapidly and uniformly not only in the plasma of the animal (28–50 ml/kg for rabbit) but also in highly perfused tissues and interstitial fluids as well in view of its major content being water-soluble low molecular mass toxins (<10 kDa) which may readily permeate the vascular endothelium [10]. As the ratio of the inter-compartmental transfer rate constants k_{12} and k_{21} for the venom approximates unity (k_{12}/k_{21} = 0.78), this means that at equilibrium (when inter-compartmental transfer rates are equal), the amount of venom antigens in both the compartments do not vary significantly. This finding corroborates with that obtained in our earlier pharmacokinetic study of N. sputatrix venom (k_{12}/k_{21} = 1) [22]. On the other hand, the large V_{d,area} of the venom (1.8 L, more than 10-fold of the total blood volume of a 2-kg rabbit) suggests that this cobra venom antigen distributes extensively to the peripheral or extra-vascular tissues. This seems to be a general phenomenon for venom antigen distribution in experimentally envenomed animals [12,13,15]. Venom toxins that are distributed widely into the peripheral compartment may be associated with the rebound phenomenon that sometimes occur during antivenom therapy as rapid immunodepletion of venom toxins in the blood favors the redistribution of venom antigens from the peripheral back into the central compartment [31,32].

To study the absorption of venom from the non-vascular parenteral administration site, the pharmacokinetics of N. sumatrana venom was studied following an intramuscular administration of the venom (sub-lethal dose) into rabbits. The resulting serum venom antigen concentration-time profile showed a relatively fast absorption of some venom antigens within the first few minutes. A subsequent short lag observed during the absorption phase of the venom was probably due to the absorption of some venom antigens via the lymphatic route from the injection site into blood circulation [20]. However, the serum concentration-time profile of the intramuscularly injected whole venom yields apparently indistinguishable absorption and distribution phases of the various toxins. The indistinguishable absorption and distribution phase reflects the continuous absorption of various antigenic venom components that occur simultaneously with their respective distribution phases. There was only one major serum concentration peak seen at 1 h, presumably caused by the summed absorption of a bulk of phospholipase A2 (T_{max}~2 h) and the three-finger toxins, i.e. neurotoxin and cardiotoxin (T_{max}~0.5 h) into the systemic circulation occurring at a rate in close proximity with each other. Rapid absorption of the venom with a short T_{max} correlates with the fast onset of neurotoxic effect in cobra envenoming, where the venom is known to exert direct inhibitory action on the neuromuscular junction via a postsynaptic blockade [33]. Administration of the venom by intramuscular route did not alter T_{1/2b}, V_{d,area} and CL of the venom antigens. This indicates that the elimination (and not the absorption) process is likely the rate-limiting step for the terminal phase of the pharmacokinetic profile of intramuscularly injected venom.

As demonstrated from ion-exchange HPLC studies [10], N. sumatrana venom contains more neurotoxin and cardiotoxin (17% and 40%, respectively) compared to N. sputatrix venom (8% neurotoxin and 35% cardiotoxin). However, N. sputatrix venom has substantially greater amount (35%) of phospholipase A2 than N. sumatrana venom (28%). This may account for the somewhat greater plasma clearance of N. sumatrana venom (91.3 ml/h) compared to that of N. sputatrix venom (68.7 ml/h), since the smaller three-finger toxins (more abundant in N. sumatrana venom) are cleared faster than the larger phospholipase A2 (more abundant in N. sputatrix venom), especially via the renal excretion route.

The bioavailability of N. sumatrana venom following i.m. injection was 41.9%, indicating incomplete absorption of the antigenic venom components from the injection site into the systemic circulation. This may be due to strong affinities of the cobra venom toxins at the injection site [12,21], and this hypothesis correlates well with cobra venom’s prominent toxic effect on local tissues that lasts for days to weeks [9,34–36]. The bioavailability of N. sumatrana venom (F_{i.m.}) was similar to that of N. sputatrix venom (F_{i.m.} = 41.7%), which is a reflection of the fact that the cardiotoxin represents the bulk of both venoms (F_{i.m.} of cardiotoxin was 46%, discussed below).

Pharmacokinetics of the individual major toxins of N. sumatrana venom

Since snake venom is a mixture of hundreds of proteins and peptides, it is therefore virtually impossible to investigate the pharmacokinetics of each individual toxin when the whole venom was administered into rabbits. As such, in this study, we only selected three representative toxins of N. sumatrana venom (neurotoxin, cardiotoxin and phospholipase A2) for pharmacokinetic investigations. These three toxins also represent the major types of toxins in the venom.

It should be noted that accurate quantitative measurement of individual toxins in the serum of experimentally envenomed animal using ELISA assay is not always feasible because of the immunological cross-reactivities observed among the snake venom toxins [37]. Indeed, our immunological cross-reaction studies revealed extensive cross-reactivity between the phospholipase A2 and polypeptide neurotoxin purified from N. sumatrana venom, demonstrating that unrelated venom proteins of distinctive primary structures and biological functions may share common antigenic domains [27,38]. As such, in the present report the pharmacokinetics of N. sumatrana venom purified phospholipase A2, neurotoxin and cardiotoxin was studied after intravenous or intramuscular injection of a sub-lethal dose of each toxin into rabbits. Double-sandwich ELISA was developed in which specific
anti-toxin IgG’s (i.e., anti-PLA$_2$ IgG, anti-NTX IgG, anti-CTX IgG) were used to measure the serum toxin antigen levels following injections of the individual toxins into rabbits.

The individual serum concentration-time profiles of the toxins, as with the whole venom, injected intravenously were also best fitted to an open two-compartment pharmacokinetic model, where the toxins distributed between central and peripheral compartments. Following intravenous administration, the individual toxins i.e, phospholipase A$_2$, neurotoxin and cardiotoxin demonstrated shorter distribution half-lives (0.36–0.66 h) compared to the whole venom (0.93 h), reflecting a more rapid distribution of the purified toxins on entering the systemic circulation.

On the other hand, unlike that observed for the whole venom, there was no fluctuation pattern during the absorption and/or distribution phase in the serum concentration-time profile of individual toxins administered intramuscularly. The significant differences in the absorption of the whole venom and toxins were also reflected by the time to reach peak concentration ($T_{\text{max}}$). The neurotoxin and cardiotoxin antigens reached their respective peak concentrations much faster than phospholipase A$_2$, indicating fast absorption of these two low molecular mass toxin molecules (approx. 8 kDa) from the injection site into the systemic circulation. These principal cobra toxins are known to directly target receptors and cellular membranes, inducing rather rapid tissue responses compared to some vipoed toxins the actions of which involve intermediate steps to accomplish the toxic effect, for instance, coagulopathy secondary to defibrinogenation induced by thrombin-like enzymes [32]. The fast absorption of neurotoxin and cardiotoxin likely accounts for the rapid onset of the systemic effects upon cobra envenomation i.e., neuromuscular paralysis and cardiac complications [7,9,39]. In view of the rapid absorption of these major toxins, meticulous monitoring for early institution of antivenom when indicated becomes crucial in order to alleviate the severity of syndrome and to preempt fatal outcome.

Furthermore, all the three toxins exhibited a large $V_{\text{d,area}}$ (1.6–2.2 L) which are >10 fald of the total blood volume of a rabbit, suggesting that the toxin antigens distributed extensively into the peripheral tissues. This finding is congruent with the large volume of distribution of the whole venom in rabbits as described above. Both the neurotoxin and cardiotoxin (2.0–2.2 L) showed a larger $V_{\text{d,area}}$ compared to the phospholipase A$_2$ (1.4 L), and this may be because low molecular mass proteins like neurotoxin and cardiotoxin (with molecular mass of approximately 7–8 kDa) cross the capillary endothelium more easily than do the larger proteins [13] such as phospholipase A$_2$ (16 kDa).

In this study, the terminal half-lives ($T_{1/2\beta}$) of neurotoxin and cardiotoxin were similar (8.6–8.8 h) but shorter than that of phospholipase A$_2$ (11.7 h). This finding is consistently reflected in the systemic clearance of the three toxins, where the clearance values of neurotoxin and cardiotoxin were significantly larger (indicative of faster elimination) than that of phospholipase A$_2$. Assuming that the elimination takes place primarily from the central compartment and probably via the renal excretion route, the faster clearance of neurotoxin and cardiotoxin can be explained by the higher vascular permeability of the two toxins as both are low molecular mass peptides. However, the $T_{1/2\beta}$ values for the neurotoxin and cardiotoxin determined in this study are substantially different from the terminal half-lives of African cobras’ $\alpha$-neurotoxin (15–29 h, in rabbits) [13] and that of cytotoxin from Chinese cobra, Naja naja atra (3.5 h, in rabbits) [12], suggesting intragenic variations in the pharmacokinetics of these cobra three-finger toxins.

Among the three major toxins, N. sumatrana neurotoxin has the most complete systemic absorption from the injection site, as evidenced by its higher intramuscular bioavailability ($F_{\text{inj}} = 81.5\%$) than that of phospholipase A$_2$ (68.6%) and cardiotoxin (45.6%). This is in agreement with the finding of Ismail et al. (1998) [16], who reported a bioavailability of 89% for V. agglutina $\alpha$-neurotoxin. Interestingly, the $F_{\text{inj}}$ of cardiotoxin was only 45.6%, presumably due to the strong binding affinity of cardiotoxin to the tissues at the injection site resulting in a poor absorption of cardiotoxin into the systemic circulation. On the other hand, the $F_{\text{inj}}$ of the phospholipase A$_2$ was 68.6%, indicating that a substantial amount of the toxin remained at the injection site. Indeed, bites from N. sumatrana (and most Naja cobras) can produce local envenomation characterized by local tissue necrosis involving the cutaneous, muscular and connective tissue layers [2,9,34,40,41]. Cardiotoxin and phospholipase A$_2$ have been reported to interact synergistically and possess potent cytolytic activity [42,43], and their substantial unabsorbed amount at the injection site seem to suggest that their toxic effects play an important role in local envenomization, which consequences include tissue necrosis following cobra bites, as well as venom ophthalmia in venom-spitted victims [44].

Although the i.e. pharmacokinetic behavior of neurotoxin is similar to that of cardiotoxin (particularly in having a rapid absorption with a short $T_{\text{max}}$), their intramuscular bioavailabilities differed markedly. The relatively low bioavailability of cardiotoxin would suggest that the systemic effects of cardiotoxin may not be that prominent in cobra envenomation, even though the venom contains relatively large amount of cardioxidins (40% of venom content [10]). Furthermore, the neurotoxin is known to be much more lethal than both the cardiotoxin and phospholipases A$_2$, with an approximate 10-fold lower $LD_{50}$ in mice (0.1 µg/g, [34]). It belongs to $\alpha$-neurotoxins with high intrinsic activity of inhibiting the motor endplate nicotinic receptors vis-à-vis that of cardiotoxins and phospholipases A$_2$, the target receptors of which are primarily different and their actions are not crucial in mediating neuromuscular paralysis - the central cobra envenomation feature that leads to rapid death [34]. This is consistent with clinical reports where rapid onset of neuromuscular paralysis (caused by neurotoxins) is the most common fatal manifestation of systemic cobra envenomation, where victims may succumb to respiratory failure and death ensued within minutes to hours [9,36,45]. The pharmacokinetic result in addition to the neuromuscular blockade activity of neurotoxin generally supports the hypothesis that the neurotoxin plays the principal role in systemic envenomation of N. sumatrana, and should be one of the most crucial toxins to be targeted by antivenom. Nevertheless, variations of neurotoxins across cobra species have been reported on their structures and activities, and the phenomenon is likely the clue to varied efficacies of commercially available antivenoms in the cross-neutralization of cobra venoms in the region [46]. The pharmacokinetic profiling method hence appears useful in validating the toxin’s role from the pharmacokinetic aspect, and may be further utilized as a tool in assessing antivenom efficacy on the targeted toxin derived from different cobras.

**Pharmacokinetics of cardiotoxin following intravenous or intramuscular administrations of whole N. sumatrana venom**

In view of the negligible immunological cross-reactivity between cardiotoxin with phospholipase A$_2$ and neurotoxin, it is possible to accurately determine the serum concentration of cardiotoxin following intravenous or intramuscular administration of the whole N. sumatrana venom using the same double-sandwich ELISA developed. This study would help to shed light on whether the pharmacokinetics of an individual toxin could be altered by other
venom constituents, and whether the information gathered from the pharmacokinetic study of individual toxins can be applied in situations where the whole venom was injected.

The serum concentration-time profile of cardiotoxin when whole venom was injected was found to be similar to that when only purified cardiotoxin was injected (Figure 3C and 3D). It is however noted that when whole venom was injected, cardiotoxin exhibited a longer $T_{1/2}$, and a lower CL than when only cardiotoxin was injected. The results therefore suggest that the rate of elimination of cardiotoxin in the whole venom is likely affected by the presence of other venom components in the venom due to competition among various venom components for the elimination processes. The results reflect that in *N. sumatrae* envenomation, pharmacokinetic characteristics of individual major toxins can be largely applied to situations where the whole venom is injected, with the possible exception that the rate of elimination of the toxins determined may be higher than that of the whole venom. On the other hand, the intramuscular bioavailability ($F_{m}$) of cardiotoxin injected with whole venom (39.5%) was similarly low, if not even lower, compared to the $F_{m}$ of cardiotoxin when only the toxin was administered (45.6%), consistent with the indication that cardiotoxin remained substantially unabsorbed at the injection tissue site.

**Conclusions**

In general, the elimination half-life of the whole venom is determined by the toxic components with the longest $T_{1/2}$.

**References**

1. Gutiérrez JM, Theakston RDG, Warrell DA (2006) Confronting the neglected problem of snake bite envenoming: The need for a global partnership. PLoS Med 3(6): e150. doi:10.1371/journal.pmed.0030150
2. WHO (2010) WHO guidelines for the production control and regulation of snake antivenom immunoglobulins. Geneva: WHO Press, World Health Organization
3. Tan KK, Choo K, Ariffin WA (1996) Snakebite in Kelantanese children: A five year experience. Toxicon 32(2): 225–230.
4. Jamaa I, Robela M, Rosalina R, Urdan RG (2004) Prevalence of snake bites in Kangar District Hospital, Perlis, West Malaysia: A retrospective study (January 1999-December 2000). Southeast Asian J Trop Med Public Health 35(4): 962–965.
5. Jamaa I, Robela M, Ng TK, Ch’ng KB, The YS, et al. (2006) Retrospective prevalence of snakebites from Hospital Kuala Lumpur (HKL) (1999-2003). Southeast Asian J Trop Med Public Health 37(1): 200–205.
6. Chew KS, Khor HW, Ahmad R, Rahman NH (2011) A five-year retrospective review of snakebite patients admitted to a tertiary university hospital in Malaysia. Int J Emerg Med 4(1): 1–6.
7. Ismail AK, Weinstein SA, Adiyaa M, Apparao P (2012) Ventricular bigeminy following a cobra envenomation. Clin Toxicol (Phila) 50(6): 518–521.
8. Wu¨ster W (1996) Taxonomic changes and toxinology: Systematic revisions of the Asiatic cobras (Naja naja species complex). Toxicon 34(6): 399–406.
9. Reid HA (1964) Cobra bites. Br Med J 2(1): 540–545.
10. Yap MKK, Tan NH, Fung SY (2011) Biochemical and toxico- logical characterization of Naja sumatrana (Equatorial spitting cobra) venom. J Venom Anim Toxins Incl Trop Environ Tox 17(4): 451–459.
11. Hung DZ, Liao MY, Lin-Shiau SY (2003) The clinical significance of venom detection in patients of cobra snakebite. Toxicon 41: 409–415.
12. Guo MP, Wang QG, Liu GF (1993) Pharmacokinetics of cytotoxin from Chinese cobra (Naja naja atra) venom. Toxicon 31: 339–343.
13. Audebert F, Utrizzera M, Sahouraud A, Schermann JM, Bon C (1994) Pharmacokinetics of Viperus ursinus venom after experimental envenomation in rabbits. J Pharmacol Toxicol 28: 152–157.
14. Barral-Netto M, von Sohsten RL (1991) Venom kinetics of Crotalus durissus tenuis venom in mice: Evidence for a rapid clearance. Toxicon 29(4–5): 527–531.
15. Ismail M, Aly MMM, Abd-Elsalam MA, Morad AM (1996) A three-component open pharmacokinetic model can explain variable toxicities of cobra venoms and their alpha toxins. Toxicon 34(9): 1101–1122.
16. Ismail M, Abd-Elsalam MA, Al-Ahmad MS (1998) Pharmacokinetics of $^{14}C$-labelled Naja haje venom and its specific antivenin: flash absorption and distribution of the venom and its toxin versus slow absorption and distribution of IgG, F(ab)2 and F(ab’)2 of the antivenin. Toxicon 36(1): 93–114.
17. Nakamura M, Kinjo K, Miyagi C, Oka U, Sunagawa M, et al. (1993) Pharmacokinetics of Habu toxin in rabbits. Toxicon 33(9): 1201–1206.
18. Palmanese N, Khoo O, Kumaz W, Omori-Satoh T, Chanhzione L, et al. (1996) Envenomation of mice by Thai cobra (Naja kaouthia) venom: tolerable venom concentration and exposure time. Toxicon 36(5): 809–812.
19. Mello SM, Lainardi A, Remo AL, Tarasiano CA, Pereira EM, et al. (2010) Renal kinetics of Bothrops alternatus (Urubu) snake venoms in rats. Toxicon 55(2–3): 470–480.
20. Paniagua D, Jiménez L, Romero C, Vergara I, Calderón A, et al. (2012) Lymphatic route of transport and pharmacokinetics of Micrurus fulvius (coral snake) venom in sheep. Lymphology 45(4): 144–153.
21. Tseng LF, Chia TH, Lee CY (1968) Absorption and distribution of $^{111}$I-labeled cobra venom and its purified toxins. Toxicon Appl Pharmacol 12: 526–535.
22. Yap MKK, Tan NH, Sim SM, Fung SY (2013) Toxicokinetics of Naja atra (Javan spitting cobra) venom following intramuscular and intravenous administrations of the venom into rabbits. Toxicon 60: 16–23.
23. Howard-Jones NA (1985) A CIOMS ethical code for animal experimentation. WHO Chronicle 39: 51–56.
24. Hudson L, Hay FC (1980) Practical Immunology. Palo Alto: Blackwell Scientific Publications.
25. Wirdon GB (1996) Hors eradish peroxidase labeling of IgG antibody. In: Walker JM, editor. The Protein Protocols Handbook. Totowa, New Jersey: Humana Press. pp. 273–274.
26. Studier FW (1957) Analysis of bacteriophage T2, early RNA’s and proteins on slab gels. J Mol Biol 79: 237–248.
27. Tan NH, Lim KK, Jiafar MI (1993) An investigation into the antigenic cross-reactivity of Ophiophagus hannah (king cobra) venom neurotoxin, phospholipase A2, hemorhagin and L-amino acid oxidase using enzyme-linked immunoabsorbent assay. Toxicon 31: 865–872.
28. Sharleg I, Yu ABC, Wu-Pong S (2005) Applied Biopharmaceutics and Pharmacokinetics. New York: McGraw-Hill.
29. Finley EL, Dillon J, Crouch RK, Sclay KL (1998) Identification of tryptophan oxidation products in bovine alpha-crystallin. Protein Sci, 7: 2391–2397.
30. Chang HC, Tsai TS, Tsai IH (2013) Functional proteomic approach to discover geographic variations of king cobra venoms from Southeast Asia and China. J Proteomics 89: 141–153.
31. Gutiérrez JM, León G, Lomonte B (2003) Pharmacokinetic-pharmacodynamic relationships of immunoglobulin therapy for envenomations. Clin Pharmacol Ther 74: 42–51.
32. Chippaux JP (2006) Snake venoms and envenomations. Florida: Krieger Publishing Company.
33. Tan NH (1983) Isolation and characterization of two toxins from the venom of the Malayan cobra (Naja atra (japanese)). Toxicon 21(2): 201–207.
34. Tan N.H. (1991). The biochemistry of venoms of some venomous snakes of Malaysia - a review. Tropical Biomedicine 8: 91–103.
35. Warrell DA (1995) Clinical toxicology of snakebite in Asia. In: Meier J, White J, editors Handbook of Clinical Toxicology of Animal Venoms and Poisons. Boca Raton (Florida): CRC Press. pp. 493–494.
36. Wongtongkam N, Wilde H, Sithi-Amorn C, Ratanabanangkoon K (2005) A study of Thai cobra (Naja kaouthia) bites in Thailand. Mil Med 170(4): 336–341.
37. Harrison RA, Wüster W, Theakston RD (2003) The conserved structure of snake venom toxins confers extensive immunological cross-reactivity to toxin-specific antibody. Toxicon 41: 441–449.
38. Stäbeli RG, Magalhães LM, Selistré-de-Araujo HS, Oliveira EB (2005) Antibodies to a fragment of the Bothrops moojeni-aminoc acid oxidase cross-react with snake venom components unrelated to the parent protein. Toxicon 46: 308–317.
39. Paul VK (1993) Animal and insect bites. In: Singh M, editor. Medical Emergencies in Children. 2nd ed. New Delhi: Sagar Publications. pp. 624–683.
40. Iddon D, Theakston RD, Ownby C L (1987) A study of the pathogenesis of local skin necrosis induced by Naja nigricollis (spitting cobra) venom using simple histological staining techniques. Toxicon 25(6): 665–672.
41. Wong OF, Lam TS, Fung HT, Choy CH (2010). Five-year experience with Chinese cobra (Naja atra)-related injuries in two acute hospitals in Hong Kong. Hong Kong Med J 16(1):36–43.
42. Tan NH, Armugam A (1990) In vivo interactions between neurotoxin, cardiotoxin and phospholipase A2 isolated from Malayan cobra (Naja naja sputatrix) venom. Toxicon 28(10): 1193–1198.
43. Fletcher JE, Jiang MS (1993) Possible mechanisms of action of cobra snake venom cardiotoxins and bee venom melittin. Toxicon 31(6): 669–695.
44. Ismail M, Al-Bekairi AM, Abd-el Salam MA (1993) The ocular effects of spitting cobras: I. The ringhals cobra (Hemachatus haemachatus) venom-induced corneal opacification syndrome. J Toxicol Clin Toxicol 31(1): 31–41.
45. Kularatne SA, Budagoda BD, Gawarammana IB, Kularatne WK (2009) Epidemiology, clinical profile and management issues of cobra (Naja naja) bites in Sri Lanka: first authenticated case series. Trans R Soc Trop Med Hyg 103(9): 924–930.
46. Leong PK, Sim SM, Fung SY, Sumana K, Sitprija V et al. (2012) Cross Neutralization of Afro-Asian Cobra and Asian Krait Venoms by a Thai Polyvalent Snake Antivenom (Neuro Polyvalent Snake Antivenom). PLoS Negl Trop Dis 6(6): e1672. doi:10.1371/journal.pntd.0001672.