CHAPTER 6

PHARMACOKINETICS OF *NAJA SPUTATRIX* (JAVA SPITTING COBRA) VENOM AND THE EFFECT OF POLYVALENT ANTIVENOM ON ITS PHARMACOKINETICS

6.1 INTRODUCTION

Snake envenomation remains an important yet neglected public health problem in tropical and subtropical countries (Gutiérrez *et al*., 2006). Antivenom is still the only proven effective therapy for envenomation (Warrell, 2010b and WHO, 2010). The existing protocols for the therapeutic uses of antivenom, however, are largely empirical (Chippaux, 1998). This is mainly due to inadequate knowledge on the pharmacokinetics of venoms/antivenoms and the pathophysiology of envenomation, although there have been some studies in the pharmacokinetics of snake venoms and the purified toxins using animal models (Audebert *et al*., 1994; Ismail *et al*., 1998; Mello *et al*., 2010; Nakamura *et al*., 1995; Pakmanee *et al*., 1998 and Zhao *et al*., 2001). Even though cobra (*Naja* sp.) is one of the commonest causes of envenomation, to date there was limited report on the pharmacokinetics of cobra venoms and there is no information regarding the bioavailability of the venom in the circulatory system following cobra bites. To fully understand the pathophysiology and to improve treatment of cobra envenomation, it is necessary to have a good understanding of the pharmacokinetics including the bioavailability of the venom as a result of envenomation.

Naja sputatrix (Javan spitting cobra) venom has been studied extensively for its biochemical and toxinological properties (Tan, 1982a, 1982b and 1983). The spitting cobra is one of the common causes of snake envenomation in Southeast Asia. In the present work, pharmacokinetics of *N. sputatrix* venom following intravenous and intramuscular administrations of the whole venom into rabbits was investigated. In addition, the effect of a polyvalent antivenom (Neuro Polyvalent Antivenom) on pharmacokinetics of the venom was studied following experimental envenomation.

6.2 METHODS

6.2.1 Production and purification of antibody IgG against *N. sputatrix* venom in rabbits

The antibody IgG against *N. sputatrix* venom was produced in rabbits according to the method described in Chapter 3 (section 3.2.3.1). Briefly, 10 μg of *N. sputatrix* venom mixed with Freund's complete adjuvant was used in the first immunization while in subsequent immunizations, 20 μg of the venom mixed with Freund's incomplete adjuvant was injected intramuscularly at multiple sites at the back of rabbit fortnightly, for 8 weeks. Anti-*N. sputatrix* IgG was purified according to the method described in Chapter 3 (section 3.2.3.2). Anti- *N. sputatrix* IgG-HRP conjugate was prepared according to the protocol described in Chapter 3 (section 3.2.3.3)

6.2.2 Determinations of the antigenic reactivity of anti-*N. sputatrix* IgG against *N. sputatrix* venom and venom toxins by Indirect ELISA assay and Western blot

6.2.2.1 Isolation of *N. sputatrix* venom toxins

The major *N. sputatrix* venom toxins (*sputatrix* phospholipase A_2-3, sputa-neurotoxin 1 and sputa-cardiotoxin A) were isolated from the venom using methods modified from previous reports (Tan, 1982a, 1982b and 1983). However, instead of the conventional ion exchanger, Resource® S ion-exchange chromatography using Shimadzu LC-20AD HPLC system was used (as described in Chapter 4, section 4.2.1). Protein concentration was measured by absorbance at 280 nm and the fractions were collected manually. Phospholipase A_2 activity and lethality of each collected fractions were determined, as described in Chapter 3 (Section 3.2.5) and Chapter 4 (section 4.2.3.6).

6.2.2.2 Indirect Enzyme-linked Immunosorbent Assay (ELISA)

Indirect ELISA was carried out as described in Chapter 3 (section 3.2.4). Briefly, 100 ng/ml of *N. sputatrix* venom or venom toxin (*sputatrix* phospholipase A_2-3, sputa-neurotoxin and sputa-cardiotoxin A) was coated on ELISA immunoplate (SPL) and incubated overnight at 4°C. This was followed by subsequent addition of rabbit anti-*N. sputatrix* IgG (dilutions of 1:500), goat anti-rabbit IgG-HRP conjugate (dilutions of 1:6000) and 100 µl of substrate o-phenylenediamine dihydrochloride (0.4 mg/ml in 0.5 M citrate-phosphate buffer containing 0.003% (v/v) H_2O_2) for colorimetric development. The enzymatic activity was stopped after 1 h by adding 50 µl of 12.5% (v/v) sulfuric acid. The absorbance at 492 nm was then measured using Bio-Rad Model 690 microplate reader.

6.2.2.3 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot (Immunoblotting)

SDS-PAGE was carried out according to the method described in Chapter 3 (section 3.2.2). Briefly, *N. sputatrix* venom (10 µg) was loaded onto the 15% SDS-PAGE gel (reducing condition) and electrophoresis was carried out at constant volt (90 V) for 2 h. After electrophoresis was completed, the polyacrylamide mini gel was removed from the gel cassette and washed twice with UltraPure™ water for 5 min each. The proteins on the polyacrylamide mini gel was transferred to a PVDF membrane (iBlot® Gel transfer stacks, PVDF, mini, Invitrogen™) assembled on iBlot® blotting system (Invitrogen™), using Program P3, 20 V for 7 min. After transfer, the PVDF membrane was rinsed twice in UltraPure™ water for 5 min each. The PVDF membrane was regenerated by immersing the membrane in 100% (v/v) methanol, followed by rinsing twice with 20 ml of UltraPure™ water for 5 min. The PVDF membrane

was later blocked in blocking buffer (2% (w/v) BSA in 1X Tris-buffered saline-Tween) for 1 h with gentle shaking on orbital shaker. The membrane was then washed 3 times with Tris-buffered saline-Tween for 5 min each, followed by incubation of anti-*N. sputatrix* IgG (dilution of 1:200 in Tris-buffered saline-Tween) for 1 h at room temperature, with gentle shaking. Subsequently, the membrane was washed with Tris-buffered saline-Tween and 10 ml of Goat anti-rabbit IgG-HRP conjugate (dilution of 1:1000) was added into the PVDF membrane and incubated for 30 min at room temperature. The membrane was washed with Tris-buffered saline-Tween, 3 times for 5 min each followed by rinsing in UltraPure™ water prior to colorimetric development. Five milliliters of substrate solution (Novex® HRP Chromogenic Substrate (TMB), Invitrogen) was added and allowed to incubate for 15 min until the desired intensity was achieved.

6.2.3 Double-sandwich Enzyme-linked Immunosorbent Assay (ELISA)

6.2.3.1 Determination of serum venom antigen levels in experimental envenomed rabbits

Double-sandwich ELISA was used to monitor the serum venom antigen levels following experimental envenomation in rabbits (n = 3). ELISA immunoplate (SPL) was incubated overnight at 4°C with 100 µl of the anti-*N. sputatrix* IgG (4 µg/ml in 0.05 M sodium carbonate, pH 9.6). The immunoplate was washed 4 times with 200 µl of PBS-Tween. The plate was then incubated with 100 µl of diluted rabbit serum samples (1:20) collected at different time intervals, for 2 h. This was followed by sequential washing with 200 µl of PBS-Tween for 4 times and incubation with 100 µl of anti-*N. sputatrix* IgG-HRP conjugate (1:400) for 2 h. The plate was again washed for 4 times with 200 µl of PBS-Tween followed

by incubation with 100 µl of substrate o-phenylenediamine dihydrochloride (0.4 mg/ml in 0.1 M citrate-phosphate buffer, pH 5 containing 0.003% (v/v) H_2O_2). The reaction was terminated 1 h later by adding 50 µl of 12.5% (v/v) sulfuric acid. The absorbance at 492 nm was then determined using Bio-Rad Model 690 microplate reader. A standard curve of varying venom antigen concentrations (2.5-15 ng/ml) was constructed using varying dilutions of venom in spiked pre-envenomed sera.

6.2.3.2 Determination of serum Neuro Polyvalent Antivenom (NPAV) levels by double-sandwich ELISA

The serum antivenom (NPAV) concentrations in rabbits were measured by double-sandwich ELISA. ELISA immunoplate (SPL) was coated overnight at 4°C with 100 µl of the rabbit anti-horse $F(ab')_2$ (4 µg/ml in 0.05 M sodium carbonate, pH 9.6). The immunoplate was washed 4 times with 200 µl of PBS-Tween. The plate was then incubated with 100 µl of serum samples (dilution of 1:20) collected at different time intervals, for 2 h. This was followed by sequential washing with 200 µl of PBS-Tween for 4 times and incubation of 100 µl of rabbit anti-horse $F(ab')_2$-HRP conjugate (1:6000) for 2 h. The plate was again washed 4 times with 200 µl of PBS-Tween followed by incubation with 100 µl of substrate o-phenylenediamine dihydrochloride (0.4 mg/ml in 0.5 M citrate-phosphate buffer containing 0.003% (v/v) H_2O_2). The reaction was terminated 1 h later by adding 50 µl 12.5% (v/v) sulfuric acid and the absorbance at 492 nm was then determined using Bio-Rad Model 690 microplate reader. A standard curve of antivenom (NPAV) concentrations was constructed using varying dilutions of NPAV (5-25 ng/ml) in spiked pre-envenomed sera.

6.2.4 Pharmacokinetics of *N. sputatrix* venom after intravenous (*i.v.*) and intramuscular (*i.m.*) administration into rabbits

A sub-lethal dose of *N. sputatrix* venom was administered intravenously (*i.v.*, 0.09 mg/kg) and intramuscularly (*i.m.*, 0.5 mg/kg) into rabbits (n = 3, approximately 2 kg). Blood samples were collected from central ear artery before experimental envenomation. Following intramuscular injection of the venom into rabbits, blood samples were collected at specific time points at 1 min, 5 min, 10 min, 20 min, 30 min, 45 min, 1 h, 2 h, 3 h, 5 h, 8 h and 24 h. For intravenous route of injection of venom, the blood samples were collected from central ear artery at 1 min, 15 min, 30 min, 1 h, 3 h, 6 h and 24 h. The collected rabbits' blood samples were centrifuged at 3,500 xg for 20 min to obtain the sera. Blood sera were kept at -20°C until further analysis. The serum antigen levels were then measured by double-sandwich ELISA as described in section 6.2.3.1 using the pre-envenomed serum samples collected from the same animal as the control.

6.2.5 The effect of Neuro Polyvalent Antivenom (NPAV) on the pharmacokinetics of *N. sputatrix* venom in experimentally envenomed rabbits

6.2.5.1 Pharmacokinetics of *N. sputatrix* venom in the presence of a single dose of NPAV was administered

A sub-lethal dose (0.5 mg/kg) of *N. sputatrix* venom was injected intramuscularly into rabbits (n = 3, approximately 2 kg each). One hour after venom injection, 4 ml of Neuro Polyvalent Antivenom (NPAV) was infused intravenously into the marginal ear vein of rabbits over 20 min. Blood samples were collected from central ear artery at specific time points from 5 min, 30 min, 1 h, 1.5 h, 2.5 h, 4.5 h, 6.5 h, 12 h, 24 h, 48 h and 72 h. The collected blood samples were centrifuged at 3,500 xg for 20 min to obtain the serum. Blood serum was kept at -20°C until further analysis. The serum antigen levels were then measured by double-sandwich ELISA as described in section 6.2.3.1 using the pre-envenomed serum samples collected from the same animal as the control.

6.2.5.2 Pharmacokinetics of *N. sputatrix* venom in the effect of repeated dosing of NPAV were administered

In the second series of experiment, an additional 2 ml of NPAV was infused intravenously into experimentally envenomed rabbits 1 h after the initial 4 ml infusion. Blood samples were collected from central ear artery at specific time points: 5 min, 30 min, 1 h, 1.33 h, 1.83 h, 2.33 h, 2.5 h, 4.5 h, 6.5 h, 12 h, 24 h, 48 h and 72 h. The collected blood samples were centrifuged at 3,500 xg for 20 min to obtain the sera. Blood sera were kept at -20°C until further analysis. The serum antigen levels were then measured by double-sandwich ELISA as described in section 6.2.3.1 using the pre-envenomed serum samples collected from the same animal as the control.

6.2.6 Pharmacokinetics of Neuro Polyvalent Antivenom (NPAV) in rabbits

Four milliliters of NPAV was infused intravenously into the marginal ear veins of rabbits. Blood samples were collected from central ear artery at specific time points from 5 min, 30 min, 1.5 h, 2.5 h, 6.5 h, 12 h, 24 h, 48 h and 72 h. The collected blood samples were centrifuged at 3,500 xg for 20 min to obtain the sera. Blood sera were kept at -20°C until further analysis. The serum concentration of NPAV was measured by double-sandwich ELISA as described in section 6.2.3.2.

6.2.7 Pharmacokinetic analysis

The pharmacokinetic parameters of *N. sumatrana* venom and venom toxins were determined using the method of Feathering (Shargel *et al.*, 2005). The area under the curve (AUC) was calculated from zero time to the last experimental time point by trapezoidal rule and extrapolated to infinity ($AUC_{0-\infty}$) according to the formula: $AUC_{0-\infty} = AUC_{0-t} + C_t/\beta$, where t is the last experimental time point and C_t represents the last serum venom concentration determined at time t. 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. The initial phase half-life ($T_{1/2\alpha}$) and terminal phase half-life ($T_{1/2\,\beta}$) were determined by the formula $T_{1/2\alpha}$ or $T_{1/2\beta} = 0.693/\ \alpha$ or β.

The distribution rate constants for the transfer between central compartment (designated as 1) and peripheral compartment (designated as 2) were calculated from the equations: $k_{21} = (A\beta + B\alpha) / (A+B)$ and $k_{12} = \alpha + \beta - k_{21} - (\alpha\beta/k_{21})$.

The other important pharmacokinetic parameters were determined as follows:

Volume of distribution by area, $V_{d,area} = CL/\beta$

Volume of central compartment, $V_c = Dose_{i.v.}/(A+B)$

Volume of peripheral compartment, $V_p = k_{12}/ k_{21} (V_c)$

Systemic clearance, CL = dose (F)/$AUC_{0-\infty}$

$F_{i.v.}$ is the intravenous bioavailability which is 1.

$F_{i.m.}$ is the intramuscular bioavailability, and was calculated as follows:

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

6.2.8 Determination of the median lethal dose (LD_{50}) of *N. sputatrix* venom

The intravenous median lethal dose (LD_{50} *i.v.*) and intramuscular median lethal dose (LD_{50} *i.m.*) of the venom was determined as described in Chapter 3 (section 3.2.5).

6.2.9 *In vitro* and *in vivo* neutralization of *N. sputatrix* venom by Neuro Polyvalent Antivenom (NPAV)

In vitro neutralization of *N. sputatrix* venom by antivenom was carried out with slight modification from the method described by Ramos-Cerrillo *et al.* (2008).

N. sputatrix venom (2.5 LD_{50} *i.v.*) was preincubated with varying dilutions of NPAV (50-200 µl) for 30 min at 37°C and the total injected volume of 300 µl. The mixture was then injected into caudal vein of mice (n = 4). A control group consisted of mice injected with a mixture of *N. sputatrix* venom (2.5 LD_{50} *i.v.*) with normal saline. The number of mice survived after 24 h was recorded. Neutralization potency of the antivenom was expressed in term of median effective dose, ED_{50} (µl antivenom/ 2.5 LD_{50} *i.v.*), which is defined as the amount of antivenom required to neutralize the venom at survival percentage of 50%. ED_{50} was calculated by Probit method (Finney, 1952).

In vivo neutralization of *N. sputatrix* venom was carried out by intramuscular injection of *N. sputatrix* venom (2.5 LD_{50} *i.m.*) into mice (n = 4) at caudal thigh

muscle, followed by intravenous injection of varying dilutions of NPAV (50-200 µl) 10 min later, after the injection of venom. The number of mice survived after 24 h was recorded. A control group consisted of mice (n = 4) was envenomed with the same minimal lethal dose of *N. sputatrix* venom, followed by intravenous injection of normal saline into the mice 10 min later. Median effective dose, ED_{50} (µl antivenom/ 2.5 LD_{50} *i.m.*), was calculated by Probit method (Finney, 1952).

The amount of venom neutralized by the reconstituted antivenom, expressed as mg/ml, is the amount of venom that is completely neutralized by one unit volume of antivenom, as calculated according to Morais *et al*. (2010).

6.2.10 Statistical analysis

Median lethal dose, LD_{50} of the venoms and ED_{50} of antivenoms are expressed as mean with 95% confidence intervals (C.I.) and were calculated using the Probit method (Finney, 1952). All data are reported as the mean ± S.D. Shapiro-Wilk test for normality was carried out to assess the normality of the data. The mean difference between two independent groups was determined by Mann-Whitney *U* test and Kruskal-Wallis H Test to compare mean differences between two or more independent groups. The level of significance was set at $p = 0.05$.

6.3 RESULTS

6.3.1 Antigenic reactivities of anti-*N. sputatrix* IgG against *N. sputatrix* venom and venom toxins

Indirect ELISA showed that anti-*N. sputatrix* IgG reacted strongly with *N. sputatrix* venom as well as the three main types of the venom toxins: sputa-neurotoxin 1, sputa-neurotoxin A and *sputatrix* PLA_2-3 (Table 6.1). SDS-PAGE showed that the bulk of the proteins are low molecular weight proteins or polypeptides (<20 kDa) and Western blot showed that anti-*N. sputatrix* IgG binds mainly to these low molecular weight proteins/polypeptides (Figure 6.1)

Table 6.1: Indirect ELISA reactions between rabbit anti-*N. sputatrix* IgG and *N. sputatrix* venom and venom toxins.

	Absorbance at 492 nm			
	***N. sputatrix* venom or venom toxins**			
	Venom	**NTX**	**CTX**	**PLA_2**
Anti-*N. sputatrix* IgG	1.02 ± 0.23	1.01 ± 0.0033	0.95 ± 0.005	0.75 ± 0.04

The ELISA immunoplate was coated with 100 ng/ml of the venom or venom toxins, and reacted with anti- *N. sputatrix* IgG (dilution of 1:500). Subsequently, goat anti-rabbit IgG-HRP conjugate was added followed by the substrate. The absorbance at 492 nm was determined after 1 h and the values shown were mean ± S.D. (n = 3). The venom toxins used were sputa-neurotoxin 1 (NTX), sputa-cardiotoxin A (CTX) and *sputatrix* PLA_2-3 (PLA).

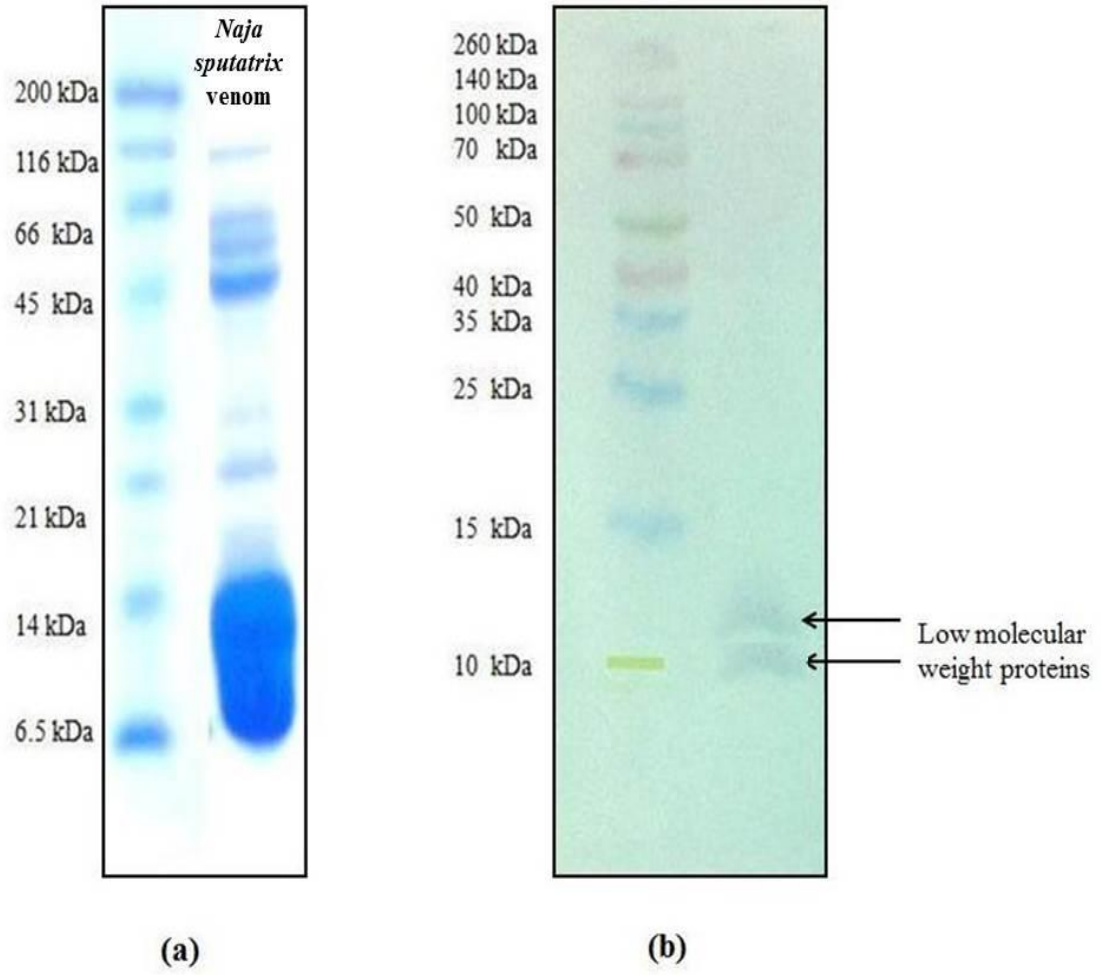

Figure 6.1: Antigenic reactivity of anti-*N. sputatrix* IgG against *N. sputatrix* venom proteins.

N. sputatrix venom was subjected to 15% SDS-PAGE (reducing condition) and stained with Coomassie Blue (a) or transferred to PVDF membrane and react with anti-*N. sputatrix* IgG (b) (Western blot).

6.3.2 Pharmacokinetics of *N. sputatrix* venom after intravenous administration

The serum concentration-time profile of whole *N. sputatrix* venom following a single *i.v.* and *i.m.* administrations into the rabbits is shown in Figure 6.2.

The *i.v.* serum concentration-time profile for *N. sputatrix* venom (0.09 mg/kg) antigen (Figure 6.2, dotted line) showed a biphasic pattern which was best fitted into an open two-compartment pharmacokinetic model with the following equation in the format of $C_t = Ae^{-\alpha t} + Be^{-\beta t}$:

$C_t = 124.17\ e^{-1.90t} + 111.06\ e^{-0.046t}$

Where C_t represents the concentration at time, t; A represents the empirical coefficient at initial phase while α represents the initial rate constant; B represents the empirical coefficient at terminal phase while β represents the terminal rate constant.

The venom antigen level declined rapidly within the first hour ($T_{1/2\alpha}$ = 0.46 ± 0.30 h), followed by a much slower decline terminal phase with a half-life ($T_{1/2\beta}$) of 15.38 ± 3.06 h. The volume of distribution by area ($V_{d,area}$) of the venom antigen in rabbits (approximately 2 kg) was 1.50 ± 0.24 L, the systemic clearance (CL) was 68.74 ± 10.67 ml/h, and the $AUC_{0-\infty}$ was 2664.96 ± 447.42 ng/ml.h (Table 6.2). The distribution rate constant for the transfer of the whole venom from the central to peripheral compartment (k_{12} = 0.94 ± 0.49 h^{-1}) was similar to that for the transfer from peripheral to central compartment (k_{21} = 0.91 ± 0.42 h^{-1}), indicating similar amount of the venom antigen are present in the central and peripheral compartments.

6.3.3 Pharmacokinetics of *N. sputatrix* venom after intramuscular administration

The intramuscular administration of crude *N. sputatrix* venom showed a more complicated serum concentration-time profile (Figure 6.2, solid line) where the absorption and distribution phases seemed to occur in an irregular pattern. The venom antigen levels peaked within 0.75 h (T_{max}, Figure 6.2) with C_{max} = 446.78 ± 10.98 ng/ml. The pharmacokinetic parameters are also shown in Table 6.2. The terminal half-life ($T_{1/2\beta}$), volume of distribution by area ($V_{d,area}$) and systemic clearance (CL) of the venom antigen following *i.m.* injection were 18.86 ± 5.61 h, 1.88 ± 0.72 L and 67.89 ± 7.05 ml/h, respectively; and these values were not significantly different from that of the *i.v.* parameters (Table 6.2). The $AUC_{0-\infty}$ for *i.m.* administration was 6193.59 ± 676.79 ng/ml.h, when 0.5 mg/kg of the venom was injected into the rabbits. However, when adjusted to the venom dosage used for *i.v.* injection (0.09 mg/kg), the "adjusted" $AUC_{0-\infty}$ of the venom antigens for *i.m.* route was only 1112.15 ± 124.93 ng/ml.h, which was substantially lower than the *i.v.* $AUC_{0-\infty}$ (2664.96 ± 447.42 ng/ml.h) values. From the two $AUC_{0-\infty}$ values, the *i.m.* bioavailability ($F_{i.m.}$) was calculated to be 41.7%, indicating only 41.7% of the venom injected intramuscularly was absorbed into the systemic circulation.

Table 6.2: Pharmacokinetic parameters following intravenous and intramuscular administrations of *N. sputatrix* venom into rabbits.

Parameters	Intravenous (*i.v.*) (LD_{50} = 0.9 µg/g)	Intramuscular (*i.m.*) (LD_{50} = 1.12 µg/g)
A (ng/ml)	124.17 ± 13.70	-
α (h^{-1})	1.90 ± 0.90	-
B (ng/ml)	111.06 ± 163.00	203.90 ± 102.73 (85.09 ± 42.87)[#]
β (h^{-1})	0.05 ± 0.01	0.04 ± 0.01
$T_{1/2\alpha}$ (h)	0.46 ± 0.30	-
$T_{1/2\,\beta}$ (h)	15.38 ± 3.06	18.86 ± 5.61
C_{max} (ng/ml)	-	446.78 ±10.98 (186.44 ± 4.58)[#]
k_{12} (h^{-1})	0.94 ± 0.49	-
k_{21}(h^{-1})	0.91 ± 0.42	-
$V_{d,\,area}$ (L)	1.50 ± 0.24	1.88 ± 0.72
V_c (L)	0.80 ± 0.09	-
V_p (L)	0.80 ± 0.17	-
CL (ml/h)	68.74 ± 10.67	67.89 ± 7.05
$AUC_{0\,-\infty}$ (ng/ml.h)	2664.96 ± 447.42	6193.59 ± 676.79 (1112.15 ± 124.93)*
Bioavailability, *F* (%)	100	41.7 ± 4.7

The sub-lethal dose of *N. sputatrix* venom injected into rabbits (n = 3, approximately 2 kg each) were *i.v.*: 0.09 mg/kg and *i.m.*:0.5 mg/kg.

Data were expressed as mean ± S.D. (n = 3)

* $AUC_{0\,-\infty}$ value was adjusted to dosage of *i.v.* injection, *i.e.* 0.09 mg/kg

[#] B and C_{max} values were adjusted to $F_{i.m.}$, *i.e.* 41.7%.

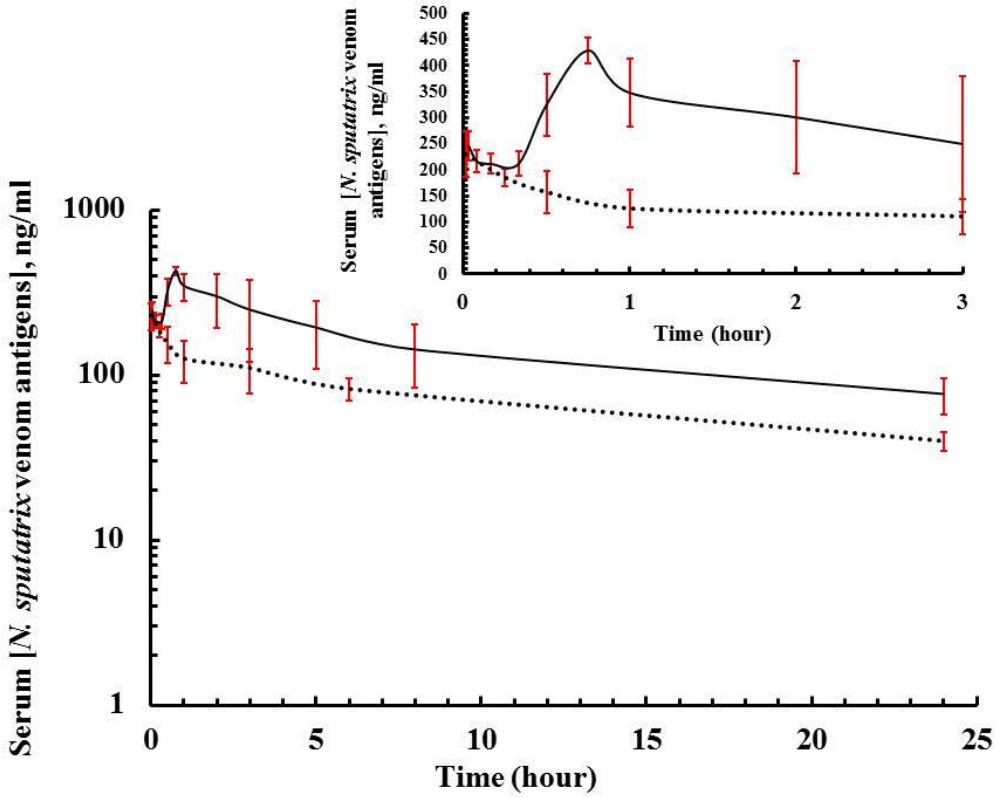

Figure 6.2: Serum concentration-time profile of *N. sputatrix* venom following intravenous and intramuscular injections of the venom (in semi-logarithmic plot).

Rabbits (approximately 2 kg) were injected intravenously and intramuscularly with a single dose of *N. sputatrix* venom. The dose for intravenous injection (••••••) was 0.09 mg/kg, and for intramuscular injection (——) 0.5 mg/kg. The serum venom antigen concentrations were determined by double-sandwich ELISA and given as the mean ± S.D. (n = 3). The insert shows serum concentration-time profile (in arithmetic plot) during the first 3 h to illustrate a clearer absorption and/or distribution phase of the venom.

6.3.4 *In vitro* and *in vivo* neutralization of *N. sputatrix* venom by Neuro Polyvalent Antivenom (NPAV)

The intravenous LD_{50} and intramuscular LD_{50} of the *N. sputatrix* venom in mice was determined to be 0.9 μg/g (0.59-1.36 μg/g) and 1.12 μg/g (0.62-1.64 μg/g), respectively. The median effective dose (ED_{50}) of NPAV was determined to be 136.72 μl/2.5 LD_{50} (*in vitro*) and 136.68 μl/2.5 LD_{50} (*in vivo*), respectively (Table 6.3), this is equivalent to 0.65 mg and 0.82 mg of the venom, respectively, neutralized by 1 ml of NPAV.

Table 6.3: *In vitro* and *in vivo* neutralization of *N. sputatrix* venom by Neuro Polyvalent Antivenom.

Neutralization	ED $_{50}$ (μl antivenom/2.5 LD_{50})	mg venom neutralized/ml antivenom
In vitro	136.72 (75.58 – 187.5 μl) / 2.5 LD_{50} *i.v.*	0.65
In vivo	138.68 (78.2 – 168.03 μl)/2.5 LD_{50} *i.m.*	0.82

Values in range for ED_{50} indicated the 95% of confidence interval (C.I.). Each group of 4 mice was used in the *in vivo and in vitro* neutralization.

6.3.5 Serum concentration-time profile of NPAV in rabbits

The serum $F(ab')_2$ concentration-time profile showed a biphasic decrease following an intravenous infusion of 4 ml of NPAV (Figure 6.3). The pharmacokinetic parameters were determined: $\alpha = 0.41 \pm 0.11\ h^{-1}$, $\beta = 0.036 \pm 0.017\ h^{-1}$, the $T_{1/2\alpha}$ and $T_{1/2\beta}$ were 1.76 ± 0.43 h and 22.42 ± 10.22 h, respectively; the k_{12} and k_{21} were $0.20 \pm 0.13\ h^{-1}$ and $0.16 \pm 0.05\ h^{-1}$, respectively. The volume of distribution by area ($V_{d,area}$) was 2.12 ± 0.95 L.

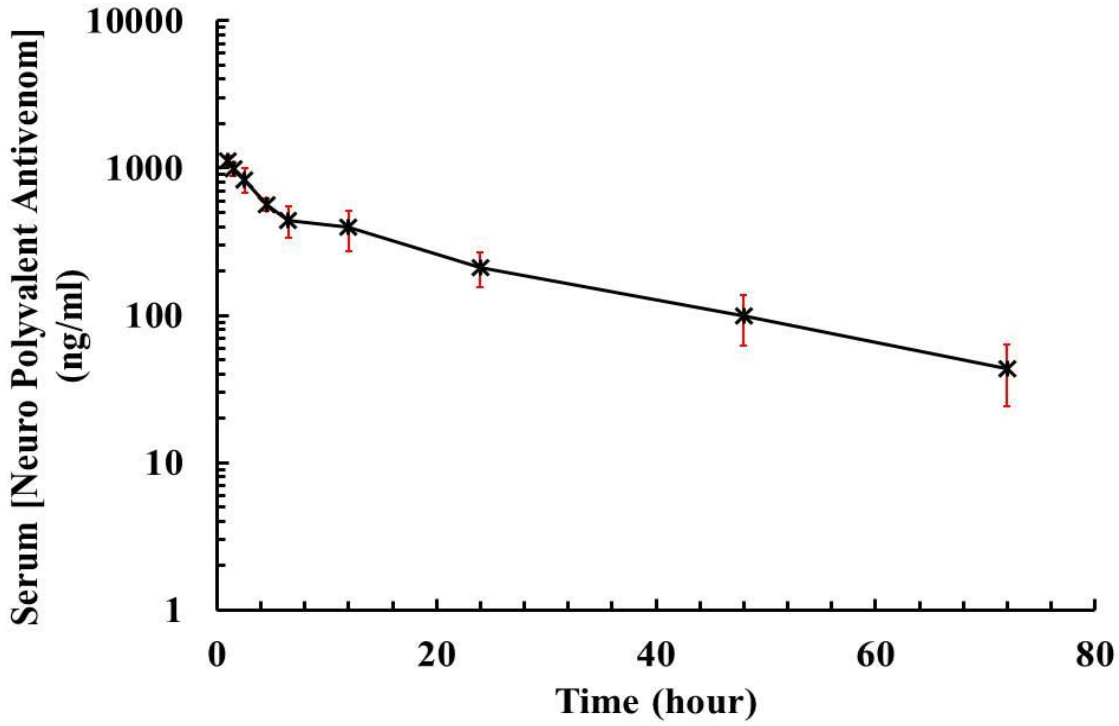

Figure 6.3: Serum concentration-time profile of the Neuro Polyvalent Antivenom (NPAV) in rabbits.

Four milliliters of Neuro Polyvalent Antivenom (NPAV) was infused into rabbits (approximately 2 kg) via the marginal ear veins of the animals. The concentration of the $F(ab')_2$ was measured by double-sandwich ELISA and the values shown are mean ± S.D. (n = 3)

6.3.6 The effect of Neuro Polyvalent Antivenom (NPAV) on the pharmacokinetics of *N. sputatrix* venom

To examine the effect of NPAV on the pharmacokinetics of *N. sputatrix* venom, 4 ml of NPAV was infused intravenously into the rabbit, over 20 min, 1 h after the *i.m.* injection of the venom (0.5 mg/kg). It was observed that, there was a sharp decline in the venom antigen level immediately after the infusion of the antivenom, from the peak 300 ng/ml to 80 ng/ml 1 h after the antivenom infusion (Figure 6.4). This was, however, followed by gradual but transient resurgence of the serum venom antigen level to about 90 ng/ml 3 h later, and that venom antigen levels persisted more than 24 h after the experimental envenomation. As a result, the 4 ml of NPAV only reduced the $AUC_{0-\infty}$ value of the venom antigens by 34% (Table 6.4).

In the second series of experiment, an additional 2 ml of NPAV was infused intravenously 1 h after the initial infusion of 4 ml of NPAV. This managed to reduce the serum venom antigen levels to <50 ng/ml after 5 h (Figure 6.4) and the $AUC_{0-\infty}$ value was reduced by 59% (Table 6.4).

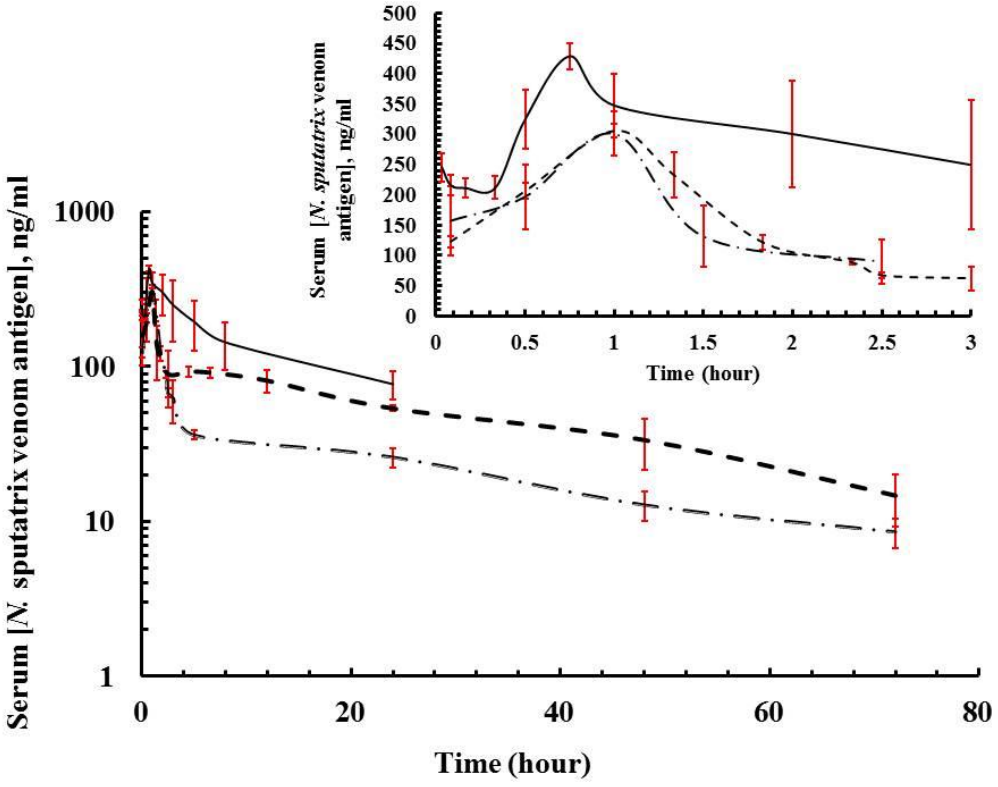

Figure 6.4: The effects of Neuro Polyvalent Antivenom (NPAV) on the serum concentration-time profile of *N. sputatrix* venom following intramuscular injection of venom (in semi-logarithmic plot).

Three sets of experiments were conducted. The first set was the control group (——) where only venom (0.5 mg/kg) was injected intramuscularly into rabbits. In the second set of experiment (— —), 4 ml of NPAV was infused into the marginal ear veins of the rabbits, 1 h after venom injection (0.5 mg/kg). In the third set of experiment (— ·), 4 ml of NPAV was infused into the marginal ear veins of the rabbits, 1 h after the injection of the venom (0.5 mg/kg), and this was then followed by infusion of another 2 ml of NPAV 1 h later. INSERT: Serum concentration-time profile of *N. sputatrix* venom (in arithmetic scale) during the first 3 h, with and without NPAV administration. All data shown are mean ± S.D. (n = 3)

Table 6.4: Area under curve ($AUC_{0-\infty}$) value in the absence and presence of antivenom immunotherapy.

Antivenom immunotherapy	$AUC_{0-\infty}$ (ng/ml. h)	Reduction in $AUC_{0-\infty}$ value (%)
In the absence of NPAV	6019.0 ± 767.7	-
4 ml of NPAV was infused	3958.2 ± 733.4	34.2
4 ml + 2 ml of NPAV was infused	2466.5 ± 182.2	59.0

Data were expressed as mean ± S.D. (n = 3). Reduction in $AUC_{0-\infty}$ value was expressed in percentage (%) of the $AUC_{0-\infty}$ in the absence of NPAV.

6.4 DISCUSSION

In this study, a low sub-lethal dose of venom was injected intravenously to avoid rabbit death. The level of venom antigen in the serum was measured as whole, using the anti-*N. sputatrix* IgG, even though snake venom is actually a mixture of many toxic components, as it is virtually impossible to monitor the serum concentration-time profile of each toxic component individually. Western blot (immunoblotting) demonstrated that the anti-*N. sputatrix* IgG raised reacted mainly with the low molecular weight toxins (MW <20 kDa) of the venom. It is well established that the low molecular weight toxins represent the bulk of the venom proteins. Indirect ELISA studies showed that the anti-*N. sputatrix* IgG reacted strongly with all three major venom components of *N. sputatrix* venom *i.e.*, neurotoxin, cardiotoxin and phospholipase A_2. Thus, the present approach represents a good approximation of the pharmacokinetics of *N. sputatrix* venom, as the anti-*N. sputatrix* IgG reacted with the bulk of the venom proteins, including all the major toxins of the venom.

The serum concentration-time profile of the venom injected intravenously can be described by an open two-compartment pharmacokinetic model where the venom was distributed between the central compartment (sometimes also known as the plasma component) and the peripheral compartment (sometimes also known as the tissue compartment). The venom was distributed to the peripheral compartment rapidly, as the half-life of distribution $T_{1/2\alpha}$ was only 0.46 ± 0.30 h. This distribution half-life value is comparable to that reported by Auderbert *et al.* (1994) for *Vipera aspis* venom (0.7 h) and by Ismail *et al*. (1996) for the African cobra venoms (22.2-30.5 min), although in the latter case, a three-compartment model was applied. The elimination half-life of the venom ($T_{1/2\beta}$ = 15.38 ± 3.06 h) was also comparable to those reported by Audebert *et al*. (1994) and Ismail *et*

al. (1996), who reported half-life of 12 h and 1130–3050 min, respectively. Furthermore, the apparent large volume of distribution by area ($V_{d,area}$, 1.5 L or 0.75 L/kg) which is approximately 10 times the rabbit's total blood volume (120-150 ml for the 2 kg rabbits) indicates that the venom antigens have also distributed substantially to extravascular tissues, and this seems to be a general phenomenon for venom antigens distribution in experimental envenomation of rabbits (Audebert *et al*., 1994; Guo *et al*., 1993 and Ismail *et al*., 1996). However, it needs to be noted that the central compartment volume is greater than the blood volume and may therefore include some other highly perfused tissues.

In an attempt to mimic the pathophysiological situation in snake envenomation, the pharmacokinetics of the venom antigen was examined following intramuscular administration of *N. sputatrix* venom in rabbits. After injection of a sub-lethal dose of venom, the serum venom antigen level peaked rapidly, indicating the relatively rapid diffusion of some of the venom antigens into the systemic blood circulation. Thereafter, it declines for a brief period before rising rapidly again to give one major serum concentration peak at about 0.75 h. This major peak is presumably associated with the absorption of the bulk of venom components consist of three-finger toxins and phospholipase A_2, whereas the irregular serum concentration-time profile during the early absorption phase may reflect the absorption of the various antigenic venom components into the systemic circulation at different rates but in close proximity to each other. Since both the absorption and distribution phases occurred in an irregular pattern, it was not possible to determine the absorption and distribution half-lives with confidence, and thus it was decided to analyze only the elimination phase. The k_{12}/k_{21} ratio of 1.0 suggests that the amount of venom antigen in the peripheral

tissue is similar to that in the central compartment (consisting of blood and highly perfused tissues) at equilibrium. The terminal half-life ($T_{1/2\beta}$), volume of distribution by area ($V_{d,area}$) and the systemic clearance (CL) of the venom following *i.m.* administration were not significantly different from the values obtained for *i.v.* route ($p > 0.05$). This suggests that the slow elimination of venom antigen was the rate-limiting step in the terminal phase, even though it is theoretically possible that continuous slow absorption of the venom components might still occur from the *i.m.* injection site, in a similar manner as when the whole venom was given by *i.v.* route. The results of this study are in contrast to the observations by Audebert *et al.* (1994), who reported a longer terminal half-life of *Vipera aspis* venom by *i.m.* compared to that of *i.v.* route, presumably caused by continuous absorption of the various venom components from the *i.m* injection site into the circulation. Thus, the terminal half-life obtained following *i.m.* injection of that viper venom probably reflected the absorption instead of elimination half-life of the venom. This is sometimes known as the "flip-flop" phenomenon in pharmacokinetics. A bioavailability of 41.7% following *i.m.* injection indicates an incomplete absorption of venom components from the site of inoculation into the systemic circulation; the residual unabsorbed toxic components may possibly be responsible for triggering the local necrotic effects. This may be caused by some cobra toxins which have strong affinities for tissues at the site of injection (Guo *et al.*, 1993 and Tseng *et al.*, 1968).

The level of bioavailability (close to 50%) is probably also the same in mice, as is demonstrated by the observed small variation in the LD_{50} (*i.v.*) and LD_{50} (*i.m.*) of the venom in mice, as well as the comparable potency of the antivenom using *in vitro* (venom and antivenom were preincubated and injected intravenously

into mice) and *in vivo* neutralization assay (venom injected intramuscularly and subsequently antivenom injected intravenously).

These results indicated that, the route of administration does not alter the terminal half-life, volume of distribution, and total body clearance of *N. sputatrix* venom, but it significantly reduced both the rate and extent of the bioavailability of the venom antigens.

The effect of NPAV on the intramuscular pharmacokinetics of *N. sputatrix* was studied following an intravenous infusion of 4 ml of NPAV 1 h after experimental envenomation. Intravenous administration of antivenom is more effective in the neutralization of the lethal and toxic effects of venom toxin (Pépin-Covatta, *et al.,* 1996), because antivenom injected intramuscularly would have slower and incomplete absorption which has no clinical significance in immunotherapy for envenomation. The serum venom antigens reached the peak concentrations within 1 h, following intramuscular injection of the venom, and thus we infused the antivenom 1 h after the experimental envenomation, which rapidly reduced the venom antigen level. However, a gradual but transient resurgence of the serum venom antigen levels was observed subsequently, suggesting a partial and transient neutralization of the circulating venom antigen. This phenomenon was also observed in a few clinical cases of Malayan pit viper (*Calloselama rhodostoma*) bites (Warrell *et al*., 1986) and a few other snake bites cases in patients (Dart *et al*., 2001; Khin Ohn Lwin *et al*., 1984 and Seifert *et al*., 1997). The venom resurgence phenomenon was not likely to be due to the mismatch of serum kinetics of the antivenom and the venom antigen, as the terminal half-life ($T_{1/2\beta}$) of the $F(ab')_2$ in serum was comparable to that of venom antigens (22.42 ± 10.22 h versus 18.86 ± 5.61 h; $p > 0.05$). Instead, it was presumably due to the redistribution of tissue-bound venom antigens into the

central compartment induced by the removal of the intravascular venom antigens (Hammoudi-Triki *et al*., 2007; Krifi *et al*., 2005 and Rivière *et al*., 1997). This phenomenon has been reported for other drugs as well (Sabouraud *et al*., 1992 and Valentine *et al*., 1994), and there were also several clinical reports on the phenomenon of venom antigens rebound after initial antivenom treatment (Dart *et al*., 2001; Rocha *et al*., 2008; Rivière *et al*., 1997 and Warrell *et al*., 1986).

The venom recurrence was successfully diminished when a 4 ml + 2 ml antivenom infusion regimen was applied. The additional 2 ml of antivenom was able to reduce the $AUC_{0-\infty}$ value by 59%, when compared to the $AUC_{0-\infty}$ value in the absence of NPAV ($p < 0.05$). The serum venom antigens level was also reduced to <10 ng/ml, a level that persists until the end of the experiment. These results support the recommendation of giving repeated doses of antivenom to the victims instead of a single bolus dose in the antivenom therapy of envenomations, and emphasize the importance to maintain a minimal effective antivenom dose at different time intervals (Maung-Maung-Thwin *et al*., 1988).

Since the bioavailabilty of *i.m.* injected venom was estimated to be 41.7%, it is estimated that 0.417 mg of *N. sputatrix* venom was absorbed into systemic circulation (total amount of venom injected *i.m.* was 1 mg). Bioavailability data indicated that the administrations of 4 ml and '4 + 2 ml' dosing regimen only managed to reduce the total amount of venom in the circulation by 34% (0.14 mg) and 59% (0.25 mg of venom), respectively. It is interesting that 1 mg of the venom injected intramuscularly (0.5 mg/kg rabbit) required 6 ml of the NPAV (4 + 2 ml NPAV) to reduce the serum venom antigen levels to <10 ng/ml, even though according to the *in vivo* neutralization assay in mice, the '4 + 2 ml' of antivenom was capable to neutralize 4.92 mg of venom. This apparent 'discrepancy' is not surprising as neutralization potency of antivenom does not

really measure the amount of venom that is *physically* neutralized but instead it is a measurement of the capability of antivenom to neutralize the *lethality* of the venom. In fact, the survived animals in either the *in vivo* or *in vitro* neutralization assay in mice may still have toxic albeit non-lethal levels of venom in the circulation. Thus, the neutralization potency of NPAV determined by *in vivo* (or *in vitro*) neutralization assay is not an adequate indicator of its capability to neutralize the toxicity of the venoms in envenomed animals and humans. The results suggest the necessity to administer a larger amount of antivenom than that which was determined by *in vivo* and *in vitro* neutralization assays in mice.