CHAPTER 7

PHARMACOKINETICS OF *NAJA SUMATRANA* (EQUATORIAL SPITTING COBRA) VENOM AND ITS INDIVIDUAL TOXINS FOLLOWING INTRAVENOUS AND INTRAMUSCULAR ADMINISTRATION OF VENOM INTO RABBITS.

7.1 INTRODUCTION

The pathophysiological and pharmacological effects arise from snake envenomation are related to the absorption and disposition of the venom toxins in the systemic circulation upon bites. There are limited pharmacokinetic studies for cobra venoms and their major toxins. To date, there were only pharmacokinetic reports of purified toxins from the venom of Formosan cobra, *Naja naja atra* (Guo *et al*., 1993 and Tseng *et al*., 1968) and a few African cobra venoms and their alpha toxins (Ismail *et al*., 1996). However, there is no information regarding the systemic bioavailability of the venom in the systemic circulation following cobra bites.

It has been reported that *N. sumatrana* venom contains predominantly basic phospholipase A_2, short alpha neurotoxins and a substantial amount of cardiotoxins. Due to the complex nature of the venoms and the fact that many venom toxins exhibited different pharmacokinetic properties, there may be variations in the onset and duration of the local and systemic symptoms in cobra envenomation patients. In the present study, double-sandwich ELISAs were developed, recruiting the monospecific antibody produced against each of the three major *N. sumatrana* venom toxins, *i.e.* basic phospholipase A_2, short neurotoxin and cardiotoxin, to measure the serum toxin concentration in a rabbit model, and thus to determine the pharmacokinetic profiles of each toxin following experimental envenomation.

7.2 METHODS

7.2.1 Isolation of *N. sumatrana* venom toxins

The major *N. sumatrana* venom toxins (phospholipase A_2, neurotoxin and cardiotoxin) were isolated from the venom by Resource® S ion-exchange chromatography using the Shimadzu LC-20AD HPLC system as described in Chapter 4 (section 4.2.1). The isolated phospholipase A_2, neurotoxin and cardiotoxin were further purified by C_{18} Reverse-phase HPLC and the protein identities were confirmed with MALDI-TOF/TOF as described in Chapter 5.

7.2.2 Production and purification of antibody IgG against *N. sumatrana* venom and venom toxins in rabbits

The antibodies IgG against *N. sumatrana* venom and venom toxins were produced in rabbits according to the method described in Chapter 3 (section 3.2.3.1). Briefly, 10 μg of *N. sumatrana* venom or 5 μg purified toxins mixed with Freund's complete adjuvant was used in the first immunization while in subsequent immunizations, 20 μg of the venom or 10 μg of purified toxins mixed with Freund's incomplete adjuvant was injected intramuscularly at multiple sites at the back of the rabbit at fortnightly for 8 weeks. The antibody IgG was purified according to the method described in Chapter 3 (section 3.2.3.2) and the antibody IgG-HRP conjugate prepared according to the protocol described in Chapter 3 (section 3.2.3.3).

7.2.3 Investigation of immunological cross-reactivity of *N. sumatrana* major venom toxins (phospholipase A_2, neurotoxin and cardiotoxin)

7.2.3.1 Indirect ELISA

Indirect ELISA assay was carried out as described in Chapter 6 (section 6.2.2.2). Briefly, ELISA immunoplate (SPL) was coated with 100 ng/ml of *N. sumatrana* venom toxin (phospholipase A_2, neurotoxin and cardiotoxin in 0.05 M sodium carbonate) and subsequently incubation with anti-PLA_2 IgG, anti-NTX IgG and anti-CTX IgG (dilutions of 1:200) and goat anti-rabbit IgG horseradish peroxidase conjugate (dilutions of 1:6000). 100 µl of the substrate o-phenylenediamine dihydrochloride (0.4 mg/ml in 0.5 M citrate-phosphate buffer containing 0.003% (v/v) H_2O_2) was added for colorimetric development and the absorbance at 492 nm was then determined using Bio-Rad Model 690 microplate reader. The degree of cross-reactivity was expressed in percentage (%).

7.2.3.2 Double-Sandwich ELISA

ELISA immunoplate (SPL) was coated overnight at 4°C with 100 µl of the anti-PLA_2 IgG, anti-NTX IgG, anti-CTX IgG (4 µg/ml in 0.05 M sodium carbonate), respectively. Plates were then incubated with 100 µl of the respective venom toxins as antigens (phospholipase A_2, neurotoxin, cardiotoxin) at concentration of 100 ng/ml. This was followed by incubation with 100 µl of anti-venom toxins IgG-HRP conjugate (1:400) for 2 h, 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) was then added. The reaction was terminated after 1 h by adding 50 µl of 12.5% (v/v) 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 (%).

7.2.3.3 Western Blot (Immunoblotting)

Ten micrograms of venom toxins (phospholipase A_2, neurotoxin and cardiotoxin) was electrophoresed on a SDS-PAGE gel (15%, reducing condition) as described in Chapter 3 (section 3.2.2). The proteins on the polyacrylamide gel were 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, as described in Chapter 6 (section 6.2.2.3).

The chromogenic detection was carried out as described in Chapter 6 (section 6.2.2.3). Anti-PLA_2 IgG, anti-NTX IgG and 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 (1:1000). Five milliliters of the substrate solution (Novex® HRP Chromogenic Substrate (TMB), Invitrogen) was added for colorimetric development.

7.2.4 Determination of serum venom antigen and toxin antigen levels using double-sandwich ELISA in experimental envenomed rabbits

Double-sandwich ELISA was used to monitor the serum venom antigen levels following experimental envenomation in rabbits (n = 3), as described in Chapter 6 (section 6.2.3.1). Briefly, ELISA immunoplates (SPL) were coated overnight at 4°C with 100 µl of the anti-*N. sumatrana* IgG (5 µg/ml in 0.05 M sodium carbonate, pH 9.6), or anti-PLA_2 IgG, anti-NTX IgG and anti-CTX IgG (4 µg/ml in 0.05 M sodium carbonate, pH 9.6), respectively. This was followed by subsequent incubation with 100 µl of diluted rabbit serum samples (1:20) collected at different time intervals, and 100 µl of anti-*N. sumatrana* IgG-

HRP conjugate (dilution of 1:500) or anti-PLA_2, anti-NTX or anti-CTX IgG-HRP conjugate (dilution of 1:400) respectively, for 2 h. Substrate o-phenylenediamine dihydrochloride (0.4 mg/ml in 0.5 M citrate-phosphate buffer containing 0.003% (v/v) H_2O_2) was added for colorimetric development. A standard curve of venom antigen concentrations was constructed using varying dilutions of venom (2.5-15 ng/ml) and toxins (0.3-10 ng/ml) in spiked pre-envenomed sera.

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

The pharmacokinetics of *N. sumatrana* venom was studied using rabbits (n = 3, approximately 2 kg). Sub-lethal dose of *N. sumatrana* venom was administered intravenously (*i.v.*, 0.1 mg/kg) or intramuscularly (*i.m.*, 0.5 mg/kg) into rabbits. Blood samples were collected from central ear artery before experimental envenomation and at specific time points (5 min, 10 min, 30 min, 1h, 2h, 3h, 6h and 24 h) after venom injection. The collected blood samples were centrifuged at 3,500 xg for 20 min to obtain the sera, which were kept at -20°C until further analysis. The serum antigen levels were then measured by double-sandwich ELISA as described in section 7.2.4 using the pre-envenomed serum sample taken from the same animal as the control.

7.2.6 Pharmacokinetics of *N. sumatrana* venom toxins (phospholipase A_2, neurotoxin and cardiotoxin) after intravenous (*i.v.*) and intramuscular (*i.m.*) administrations

The pharmacokinetics of *N. sumatrana* venom toxins in rabbits (n = 3, approximately 2 kg) was studied. Sub-lethal dose of venom toxin (phospholipase A_2 *i.m.* = 0.1 mg/kg or *i.v.* = 0.05 mg/kg; neurotoxin *i.m.* = 0.07 mg/kg or *i.v.* = 0.05 mg/kg; cardiotoxin *i.m.* = 0.15 mg/kg or *i.v.* 0.05 mg/kg) was administered into the rabbit. Blood samples were collected from 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, 10 h and 24 h) after venom injection. The collected blood samples were centrifuged at 3,500 xg for 20 min to obtain the sera. The serum antigen levels were then measured by double-sandwich ELISA as described in section 7.2.4, using the pre-envenomed serum samples collected from the same animal as the control.

7.2.7 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:

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

7.2.8 Statistical analysis

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 $p = 0.05$.

7.3 RESULTS

7.3.1 Pharmacokinetics of *N. sumatrana* venom after 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 7.1.

The *i.v.* serum concentration-time profile of *N. sumatrana* venom (0.1 mg/kg) showed a biexponential pattern which was best fitted to a two-compartment model of pharmacokinetics with the following equation in the format of $C_t = Ae^{-\alpha t} + Be^{-\beta t}$:

$$C_t = 100.96\ e^{-0.88t} + 100.28\ e^{-0.051t}$$

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

The venom antigen level declined rapidly within the first 1 h ($T_{1/2\alpha}$ = 0.77 ± 0.25 h) during initial phase followed by a much slower decline at the terminal phase ($T_{1/2\beta}$ = 13.64 ± 1.07 h). The volume of distribution by area ($V_{d,\ area}$) of the venom antigens in rabbits was 1.79 ± 0.03 L, and the systemic clearance (CL) was 91.30 ± 7.84 ml/h, and the $AUC_{0-\infty}$ was 2201.16 ± 185.47 ng/ml.h. The distribution rate constant for transfer from central to peripheral compartment (k_{12} = 0.36 ± 0.21 h^{-1}) was comparable to transfer from the peripheral to central compartment (k_{21} = 0.46 ± 0.18 h^{-1}; $p > 0.05$). This is consistent with the volume of central compartment (V_c = 0.99 ± 0.09 L) being not significantly different from that of peripheral compartment (V_p = 0.77 ± 0.16 L; $p > 0.05$).

7.3.2 Pharmacokinetics of *N. sumatrana* venom after intramuscular administration

The intramuscular administration of whole *N. sumatrana* venom in rabbits produced serum concentration-time profile (Figure 7.1, solid line) with the absorption and distribution phase appeared indistinguishable. The venom antigen levels peaked within 1 h at a concentration (C_{max}) of 391.71 ± 48.46 ng/ml. The terminal half-life ($T_{1/2\beta}$), volume of distribution by area ($V_{d,area}$) and the systemic clearance (CL) of the venom antigen following *i.m.* injection were 12.49 ± 0.89 h, 1.70 ± 0.14 L and 94.84 ± 12.74 ml/h, respectively, which were not statistically significant different from that of *i.v.* pharmacokinetic parameters ($p > 0.05$).

The $AUC_{0-\infty}$ of *N. sumatrana* venom when injected intramuscularly (0.5 mg/kg) was 4617.83 ± 583.84 ng/ml.h. However, when adjusted to the intravenous venom dose (0.1 mg/kg), the "adjusted" $AUC_{0-\infty}$ of the venom antigens following *i.m.* administration was 923.57 ± 116.77 ng/ml.h, which was significantly lower than the *i.v.* $AUC_{0-\infty}$ value (*i.e.* 2201.16 ± 185.47 ng/ml.h; $p < 0.05$). The *i.m.* bioavailability ($F_{i.m.}$) calculated from the two $AUC_{0-\infty}$ values were found to be 41.9%.

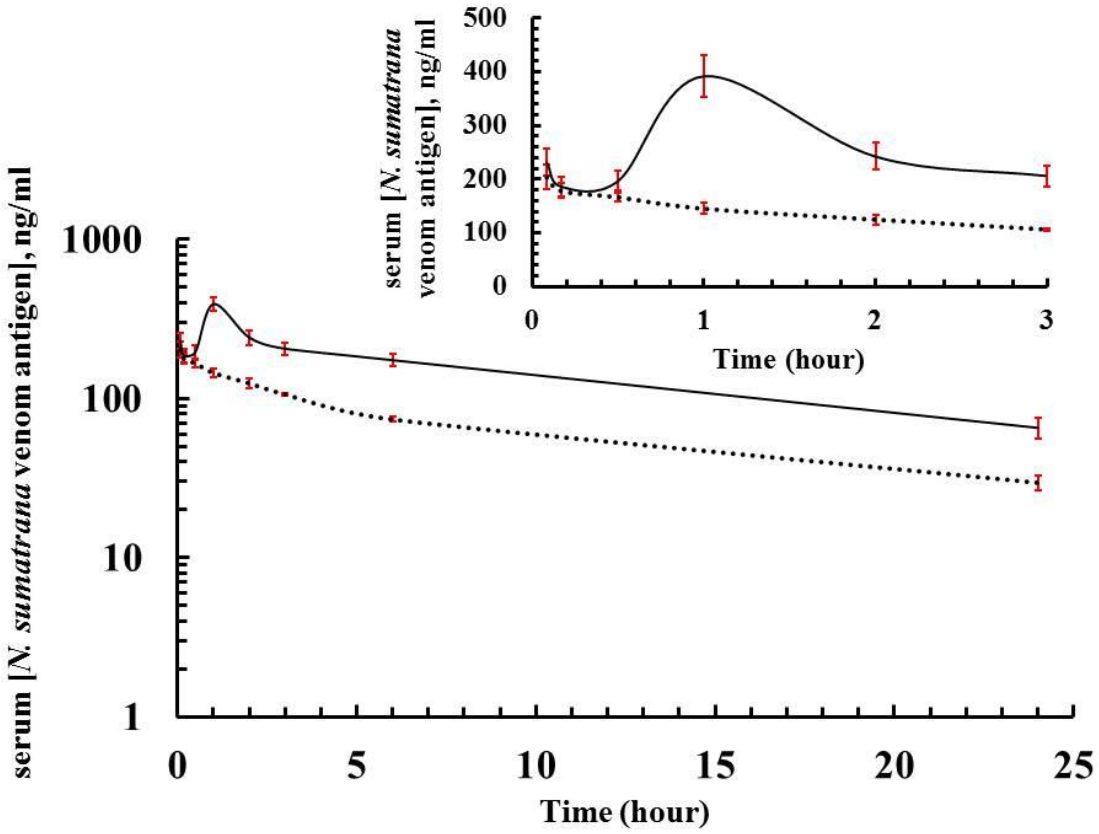


Figure 7.1: Serum concentration-time profile of *N. sumatrana* venom following intravenous (*i.v.*) and intramuscular (*i.m.*) injection of the venom(in semi-logarithmic plot).

Rabbits (approximately 2 kg) were injected intravenously and intramuscularly with a sub-lethal dose of *N. sumatrana* venom. The dose for intravenous injection (⋯⋯) was 0.1 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 means ± S.D. (n = 3). The insert shows the serum concentration-time profile of *N. sumatrana* venom following intramuscular injection of venom (in arithmetic plot) during the first 3 h.

Table 7.1: Pharmacokinetic parameters of *N. 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)	100.96 ± 17.44	-
α (h^{-1})	0.88 ± 0.39	-
B (ng/ml)	100.28 ± 1.43	247.73 ± 33.07 (103.69 ± 13.84)[#]
β (h^{-1})	0.051 ± 0.004	0.056 ± 0.004
$T_{1/2\,\alpha}$ (h)	0.77 ± 0.25	-
$T_{1/2\,\beta}$ (h)	13.64 ± 1.07	12.49 ± 0.89
C_{max} (ng/ml)	-	391.71 ± 48.46 (164.97 ± 20.29)[#]
k_{12} (h^{-1})	0.36 ± 0.21	-
k_{21}(h^{-1})	0.46 ± 0.18	-
$V_{d,area}$ (L)	1.79 ± 0.03	1.70 ± 0.14
V_c (L)	0.99 ± 0.09	-
V_p (L)	0.77 ± 0.16	-
CL (mL/h)	91.30 ± 7.84	94.84 ± 12.74
$AUC_{0-\infty}$ (ng/ml.h)	2201.16 ± 185.47	4617.83 ± 583.84 (923.57 ± 116.77)*
Bioavailability, *F* (%)	100	41.9 ± 0.2

The 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.

* $AUC_{0-\infty}$ value was adjusted to the dosage of *i.v.* injection, *i.e.* 0.1 mg/kg.
[#] B and C_{max} value were adjusted to $F_{i.m.}$, *i.e.* 41.9%.

7.3.3 Immunological cross-reactions of *N. sumatrana* venom toxins (phospholipase A_2, neurotoxin and cardiotoxin)

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 as described in Chapter 5, shown in Table 7.2.

Indirect ELISA and double-sandwich ELISA demonstrated extensive cross-reactions between phospholipase A_2 and neurotoxin (>50%), but not between these two toxins and cardiotoxin (Table 7.3).

This finding was supported by Western blot results (Figure 7.2): the anti-PLA_2 IgG only reacted immunologically with the phospholipase A_2 and neurotoxin, but not with cardiotoxin; and similarly, the anti-NTX IgG only reacted with the neurotoxin and phospholipase A_2, but not with cardiotoxin; whereas anti-CTX IgG only reacted immunologically with cardiotoxin, but not with either phospholipase A_2 or neurotoxin.

Table 7.2: MALDI-TOF/TOF identification of phospholipase A_2, neurotoxin and cardiotoxin.

***Naja sumatrana* venom toxins**	**Matched peptide sequence**	**Accession No.**
Phospholipase A_2	SWWHFADYGCYCGR GGSGTPVDDLDR CCQIHDNCYNEAEK CWPYFK TYSYECSQGTLTCK GGNNACAAAVCDCDR	Q92084 Neutral Phospholipase A_2-A
Neurotoxin	LECHDQQSSQTPTTTGCSGGETNCYK NGIEINCCTTDR	Q9PSN6 Short neurotoxin
Cardiotoxin	LVPLFYK MFMVATPK RGCIDVCPK GCIDVCPK YVCCNTDR	P60302 Cardiotoxin 3

Table 7.3: Immunological cross-reactivity of *N. sumatrana* venom toxins by Indirect ELISA and Double Sandwich ELISA.

INDIRECT ELISA		DOUBLE SANDWICH ELISA	
Venom Toxins	**% cross-reactivity**	**Venom Toxins**	**% cross-reactivity**
Anti-PLA_2 IgG		anti-PLA_2 IgG	
PLA_2	100	PLA_2	100
NTX	155.93 ± 26.73	NTX	59.35 ±13.38
CTX	0.79 ± 0.31	CTX	0
anti-NTX IgG		anti-NTX IgG	
PLA_2	55.13 ± 2.22	PLA_2	72.48 ±10.05
NTX	100	NTX	100
CTX	0.84 ± 0.18	CTX	0.72 ± 0.29
Anti-CTX IgG		anti-CTX IgG	
PLA_2	0	PLA_2	0
NTX	3.20 ± 0.43	NTX	0
CTX	100	CTX	100

For Indirect ELISA, the ELISA immunoplate was coated with 100 ng/ml of the venom toxins as antigens, and reacted with anti-PLA_2 IgG, anti-NTX IgG and anti-CTX IgG (dilution of 1:200). This was followed by incubation of goat anti-rabbit IgG horseradish peroxidase conjugate and subsequently substrate o-phenyldiamine dihydrochloride was added for colorimetric detection at 492 nm.

For Double sandwich ELISA, the ELISA immunoplate was coated with 4 µg/ml of anti-PLA_2 IgG, anti-NTX IgG and anti-CTX IgG, and then subsequently incubation with 100 ng/ml of venom antigens, and incubation of goat anti-rabbit IgG horseradish peroxidase conjugate and subsequently substrate o-phenyldiamine dihydrochloride was added for colorimetric detection at 492 nm.

The venom toxins used were phospholipase A_2 (PLA_2), neurotoxin (NTX) and cardiotoxin (CTX). Data were expressed as mean ± S.D. for n = 3.

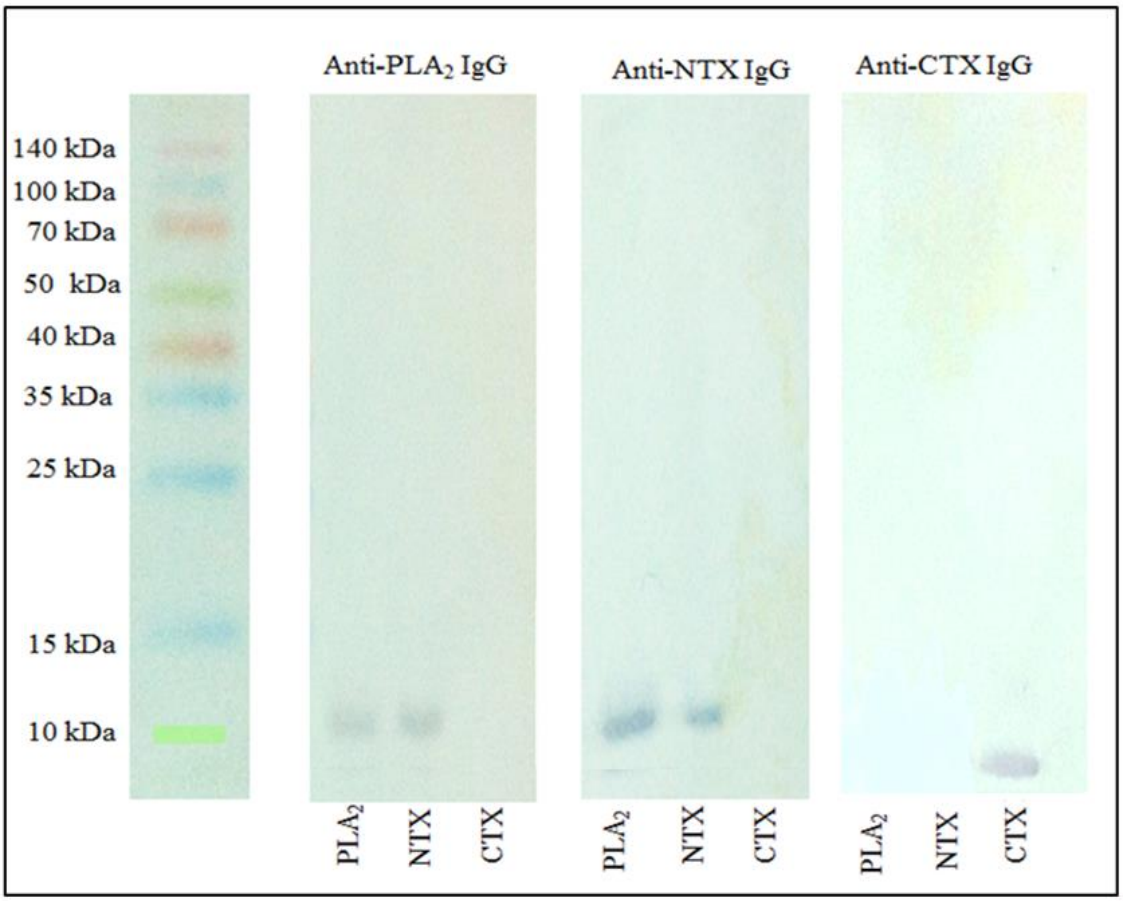


Figure 7.2: Western blot analysis (Immunoblotting).

Venom toxins (10 µg each of phospholipase A_2, 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-PLA_2 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.

7.3.4 Pharmacokinetics of phospholipase A_2 after intravenous administration

The serum concentration-time profiles of phospholipase A_2 following a single *i.v.* and *i.m.* administrations of venom into rabbits (n = 3) are shown in Figure 7.3(A).

The *i.v.* serum concentration-time profile of phospholipase A_2 showed a biexponential pattern which was best fitted to a two-compartment pharmacokinetic of the format $C_t = Ae^{-\alpha t} + Be^{-\beta t}$ represented by the following equation:

$$C_t = 141.67\ e^{-1.06t} + 52.55\ e^{-0.059t}$$

After intravenous administration, the phospholipase A_2 antigens level decreased rapidly with a distribution half-life ($T_{1/2\alpha}$) of 0.66 ± 0.03 h during the initial phase and followed by a declining terminal phase with half-life ($T_{1/2\beta}$) of 11.71 ± 0.77 h. The volume of distribution by area ($V_{d,area}$) of phospholipase A_2 antigens in rabbits was 1.62 ± 0.03 L, systemic clearance (CL) was 95.82 ± 4.80 ml/h and the $AUC_{0-\infty}$ value was 1045.41 ± 52.64 ng/ml.h.

The distribution rate constant for transfer from central to peripheral compartment (k_{12} = 0.60 ± 0.02 h^{-1}) was significantly higher ($p < 0.05$) than that of transfer from peripheral to central compartment (k_{21} = 0.33 ± 0.04 h^{-1}). Similarly, the volume of peripheral compartment (V_p = 0.94 ± 0.07 L) was larger than the volume of central compartment (V_c = 0.51 ± 0.02 L; $p < 0.05$).

7.3.5 Pharmacokinetics of phospholipase A_2 after intramuscular administration

The intramuscular administration of phospholipase A_2 (0.1 mg/kg) into rabbits peaked at 2 h (T_{max}) with a concentration of 226.64 ± 5.52 ng/ml (C_{max}) (Figure 7.3A, solid line).

The key *i.m* pharmacokinetic parameters are summarized in Table 7.4 were found to be not significantly different from the corresponding *i.v.* pharmacokinetics parameters ($p > 0.05$).

The intramuscular bioavailability ($F_{i.m.}$) of phospholipase A_2 was estimated to be 68.6% when comparing the dose-adjusted intramuscular $AUC_{0-\infty}$ of phospholipase A_2 to the intravenous $AUC_{0-\infty}$.

7.3.6 Pharmacokinetics of neurotoxin after intravenous administration

The serum concentration-time profile of neurotoxin following intravenous route of administration (0.05 mg/kg) showed a biexponential pattern that was best fitted to a two-compartment pharmacokinetic model of pharmacokinetics, as shown in Figure 7.3(B) (dotted line), and represented by pharmacokinetic equation:

$C_t = 67.49\ e^{-1.38t} + 43.96\ e^{-0.079t}$

Following intravenous administration of neurotoxin, the antigen levels declined quickly within the first 30 min with an initial distribution half-life ($T_{1/2\alpha}$) of 0.52 ± 0.11 h and a terminal eimination half-life ($T_{1/2\beta}$ = 8.75 ± 0.87 h) which is faster ($p < 0.05$) compared to whole *N. sumatrana* venom (Tables 7.1 and 7.4).

The volume of distribution by area ($V_{d,area}$), systemic clearance (CL) and the $AUC_{0-\infty}$ values of neurotoxin injected intravenously into rabbits were 2.07 ± 0.25 L, 164.11 ± 11.56 ml/h and 611.38 ± 43.33 ng/ml.h, respectively.

The transfer rate constant of neurotoxin from central to peripheral compartment (k_{12} = 0.68 ± 0.13 h^{-1}) was similar to that from peripheral compartment to central compartment (k_{21}= 0.59 ± 0.11 h^{-1}). The volume of central compartment (V_c = 0.90 ± 0.09 L) was comparable ($p > 0.05$) to the volume of peripheral compartment (V_p = 1.04 ± 0.11 L).

7.3.7 Pharmacokinetics of neurotoxin after intramuscular administration

The serum neurotoxin antigen level-time profile when the neurotoxin was injected intramuscularly (0.07 mg/kg) into rabbits was shown in Figure 7.3(B) (solid line). A very rapid absorption of the neurotoxin was observed, peak concentration (C_{max} = 120.71 ± 2.37 ng/ml) was reached within 30 min (T_{max}).

The terminal half-life ($T_{1/2\beta}$) of neurotoxin was much shorter ($p < 0.05$) than those of *N. sumatrana* venom and phospholipase A_2 (Table 7.1 and 7.4). The intramuscular bioavailability ($F_{i.m.}$) of the neurotoxin was 81.5%.

7.3.8 Pharmacokinetics of cardiotoxin after intravenous administration

The serum cardiotoxin concentration-time profile (Figure 7.3C, dotted line) after intravenous administration of the cardiotoxin (0.05 mg/kg) exhibited a typical bioexponential pattern that was best fitted to two-compartment model of pharmacokinetics, with the equation:

$C_t = 63.61\ e^{-1.25t} + 41.36\ e^{-0.081t}$

Figure 7.3(C) shows that the serum cardiotoxin antigen level decreased rapidly within 30 min with an initial distribution half-life ($T_{1/2\alpha}$) of 0.56 ± 0.08 h and terminal elimination half-life ($T_{1/2\beta}$) of 8.59 ± 0.12 h. The values of these parameters were comparable ($p > 0.05$) to those of neurotoxin, but the rate of elimination (as indicated by β values) was significantly higher than those of *N. sumatrana* venom and phospholipase A_2 ($p < 0.05$).

The volume of distribution by area ($V_{d,area}$), systemic clearance (CL) and the $AUC_{0-\infty}$ values of cardiotoxin injected intravenously into rabbits were 2.15 ± 0.05 L, 173.73 ± 2.12 ml/h and 575.67 ± 7.02 ng/ml.h, respectively.

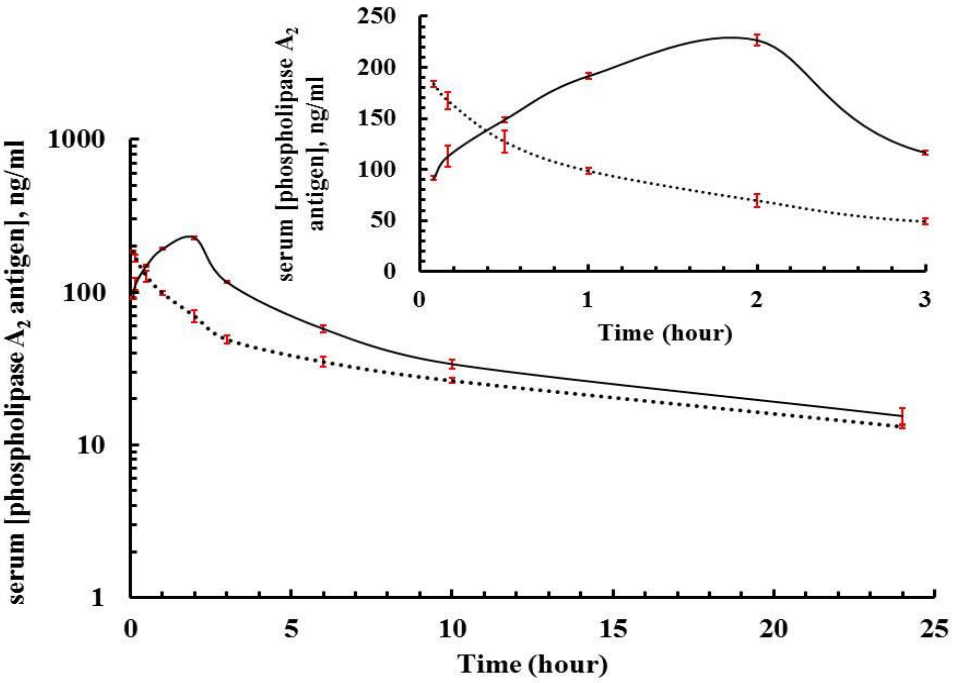
The transfer rate constant of cardiotoxin from central to peripheral compartment (k_{12} = 0.60 ± 0.12 h^{-1}) was similar ($p > 0.05$) to that from peripheral compartment to central compartment (k_{21} = 0.56 ± 0.07 h^{-1}).

7.3.9 Pharmacokinetics of cardiotoxin after intramuscular administration

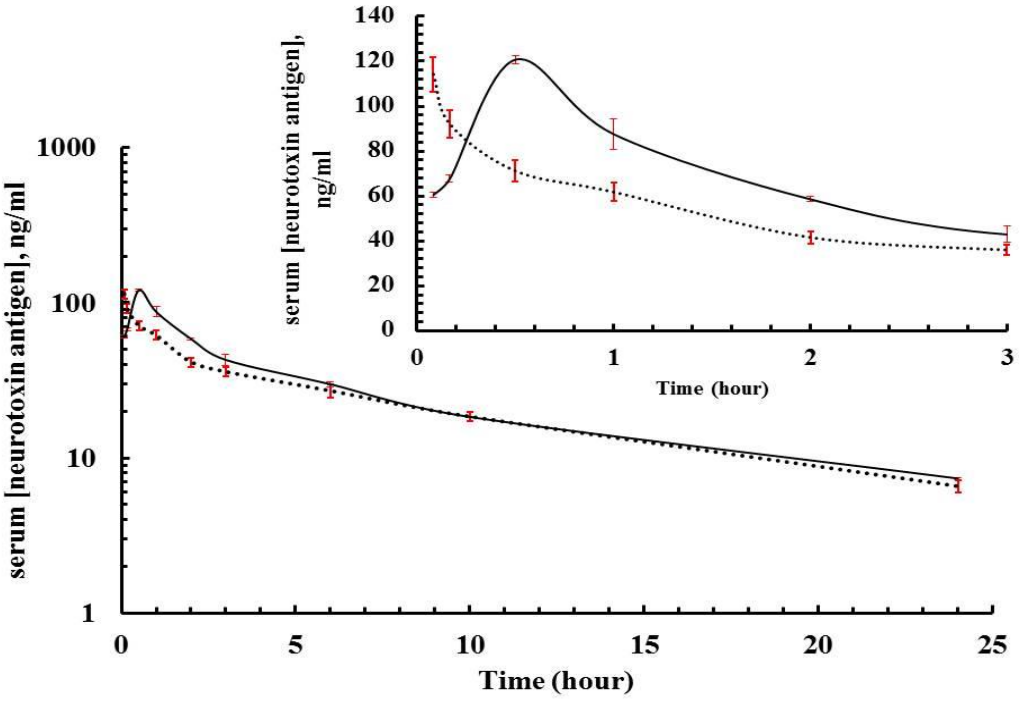
Serum concentration-time profile of cardiotoxin following intramuscular administration (0.15 mg/kg) showed a similar pattern as that of neurotoxin (Figure 7.3C, solid line), where the peak concentration, C_{max} = 132.95 ± 5.69 ng/ml. was reached within 30 min.

As with neurotoxin, the terminal half-life of cardiotoxin ($T_{1/2\beta}$ = 8.15 ± 0.10 h) following intramuscular injection, was somewhat shorter ($p < 0.05$) than that of *N. sumatrana* venom ($T_{1/2\beta}$ = 12.49 ± 0.89 h). The key pharmacokinetic parametes ($T_{1/2\beta}$, CL, $V_{d,area}$) of cardiotoxin injected intramuscularly in rabbits (Table 7.4) were not significantly different from the corresponding values when the toxin was injected intravenously. The intramuscular bioavailability ($F_{i.m.}$) of cardiotoxin was 45.6%.

(A)


serum [phospholipase A2 antigen], ng/ml
1000
100
10
1
0
5
10
15
20
25
Time (hour)
serum [phospholipase A2 antigen], ng/ml
250
200
150
100
50
0
0
1
2
3
Time (hour)

(B)


serum [neurotoxin antigen], ng/ml
1000
100
10
1
0
5
10
15
20
25
Time (hour)
serum [neurotoxin antigen], ng/ml
140
120
100
80
60
40
20
0
0
1
2
3
Time (hour)

(C)

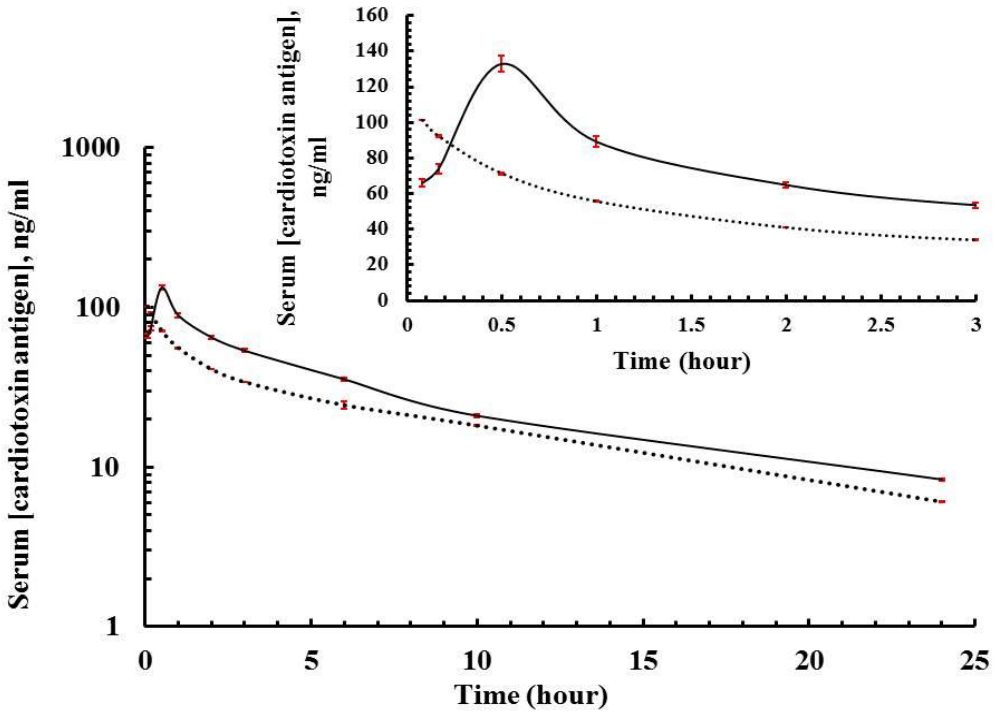


Figure 7.3: Serum concentration-time profiles of (A) *N. sumatrana* venom phospholipase A_2, (B) neurotoxin and (C) cardiotoxin; following intravenous and intramuscular injections of the respective toxin.

The serum toxin-antigen concentrations following intravenous **(⋯⋯)** and intramuscular **(——)** injection of venom were determined by respective double sandwich ELISA. Data are given as the means ± S.D. (n = 3). The insert shows serum concentration-time profile of venom toxins (in arithmetic plot) during the first 3 h.

Table 7.4: Pharmacokinetic parameters of *N. sumatrana* venom toxins (phospholipase A_2, neurotoxin and cardiotoxin) following intravenous and intramuscular administration of the venom toxins into rabbits.

Venom toxins	Phospholipase A_2		Neurotoxin		Cardiotoxin	
Parameters	Intravenous (*i.v.* 0.05 mg/kg)	Intramuscular (*i.m.* 0.1 mg/kg)	Intravenous (*i.v.* 0.05 mg/kg)	Intramuscular (*i.m.* 0.07 mg/kg)	Intravenous (*i.v.* 0.05 mg/kg)	Intramuscular (*i.m.* 0.15 mg/kg)
A (ng/ml)	141.67 ± 9.56	-	67.49 ± 6.24	-	63.61 ± 4.15	-
α (h^{-1})	1.06 ± 0.06	-	1.38 ± 0.26	-	1.25 ± 0.20	-
B (ng/ml)	52.55 ± 3.90	77.7 ± 5.09 (53.30 ± 3.49)[#]	43.96 ± 5.43	48.81 ± 4.20 (39.78 ± 3.42)[#]	41.36 ± 1.26	59.87 ± 2.20 (27.27 ± 1.00)[#]
β (h^{-1})	0.059 ± 0.004	0.069 ± 0.008	0.079 ± 0.008	0.081 ± 0.005	0.081 ± 0.001	0.085 ± 0.001
$T_{1/2\,\alpha}$ (h)	0.66 ± 0.03	-	0.52 ± 0.11	-	0.56 ± 0.08	-
$T_{1/2\,\beta}$(h)	11.71 ± 0.77	10.18 ± 1.18	8.75 ± 0.87	8.58 ± 0.53	8.59 ± 0.12	8.15 ± 0.10
C_{max} (ng/ml)		226.64 ± 5.52 (155.48 ± 3.79)[#]		120.71 ± 2.37 (98.38 ± 1.93)[#]		132.95 ± 5.69 (60.63 ± 2.59)[#]
k_{12} (h^{-1})	0.60 ± 0.02	-	0.68 ± 0.13	-	0.60 ± 0.12	-
k_{21}(h^{-1})	0.33 ± 0.04	-	0.59 ± 0.11	-	0.56 ± 0.07	-
$V_{d,area}$ (L)	1.62 ± 0.03	1.39 ± 0.24	2.07± 0.25	2.16 ± 0.15	2.15 ± 0.05	2.04 ± 0.03
V_c(L)	0.51 ± 0.02	-	0.90 ± 0.09	-	0.95 ± 0.05	-
V_p(L)	0.94 ± 0.07	-	1.04 ± 0.11	-	1.06 ± 0.02	-
CL(ml/h)	95.82 ± 4.80	95.78 ± 4.80	164.11 ± 11.56	164.11 ± 11.56	173.73 ± 2.12	173.71 ± 0.001
$AUC_{0-\infty}$ (ng/ml.h)	1045.41± 52.64	1499.90 ±154.03 (749.95 ± 77.02)*	611.38 ± 43.33	694.91 ± 2.40 (496.17 ± 1.70)*	575.67 ± 7.02	788.28 ± 23.91 (262.73 ± 7.97)*
Bioavailability, *F* (%)	100	68.6 ± 0.8	100	81.5 ± 0.6	100	45.6 ± 0.1

Data were expressed as mean ± S.D. for n = 3. * $AUC_{0-\infty}$ values were adjusted to the dosage of *i.v.* injection, *i.e.* 0.05 mg/kg for phospholipase A_2, neurotoxin and cardiotoxin. [#] B and C_{max} value were adjusted to $F_{i.m.}$, *i.e.* 68.6% (phospholipase A_2), 81.5% (neurotoxin) and 45.6% (cardiotoxin).

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

Serum concentrations of cardiotoxin following the intravenous and intramuscular administrations of whole *N. sumatrana* venom were measured by anti-CTX IgG in double-sandwich ELISA. The serum concentration-time profile of cardiotoxin when whole venom was injected was found to be very similar to when only cardiotoxin was injected (Figure 7.3C and 7.4). The pharmacokinetic parameters of cardiotoxin in whole venom following the intravenous and intramuscular administrations of venom are shown in Table 7.5. The pharmacokinetic parameters of cardiotoxin when only the toxin was administered are also listed for comparison.

Since cardiotoxin constituted 40% (Chapter 4) by dry weight of *N. sumatrana* venom, it was estimated that the equivalent dosages of cardiotoxin following intravenous and intramuscular injection of the venom (0.1 mg/kg and 0.5 mg/kg of venom, respectively) were approximately 0.04 mg/kg and 0.2 mg/kg, respectively.

The serum cardiotoxin antigens levels after intravenous administration showed a typical biphasic patterns (Figure 7.4) that best fitted a two-compartment model of pharmacokinetics, with the following equation:

$$C_t = 61.33\ e^{-1.43\ t} + 39.13\ e^{-0.063t}$$

Following an *i.v.* injection of the whole venom (0.1 mg/kg), the serum cardiotoxin levels decreased rapidly with an initial half-life ($T_{1/2\alpha}$) of 0.48 ± 0.01 h that was only slightly lower ($p > 0.05$) than the intravenous $T_{1/2\alpha}$ of cardiotoxin when only the toxin was administered. However, the $T_{1/2\beta}$ of cardiotoxin when whole venom was injected intravenously was significantly

higher than that when cardiotoxin was injected alone. This increase in $T_{1/2\beta}$ was accompanied by a decrease in CL and a non-significant difference in the $V_{d,area.}$ (Table 7.5).

The transfer rate constant of cardiotoxin from central to peripheral compartment (k_{12} = 0.75 ± 0.01 h^{-1}) was slightly higher than that of peripheral compartment to central compartment (k_{21} = 0.60 ± 0.02 h^{-1}; $p < 0.05$). The volume of central compartment (V_c = 0.77 ± 0.01 L) was also significantly smaller than the volume of peripheral compartment (V_p = 0.99 ± 0.04 L; $p < 0.05$).

Serum concentration-time profile of cardiotoxin when venom was injected intramuscularly (Figure 7.4, solid line) exhibited a similar pattern as when only cardiotoxin was injected (Fgure 7.3C, solid line) where the maximum concentration (C_{max} = 169.35 ± 8.45 ng/ml) was also reached within 30 min (T_{max}).

The dose-adjusted intramuscular $AUC_{0-\infty}$ value of cardiotoxin when whole venom was injected was substantially lower than the *i.v.* $AUC_{0-\infty}$ value ($p < 0.05$). The intramuscular bioavailability ($F_{i.m.}$) of cardiotoxin when whole venom was injected was 39.5%, which was only slightly lower than the $F_{i.m}$ of cardiotoxin when only cardiotoxin was injected (45.6%).

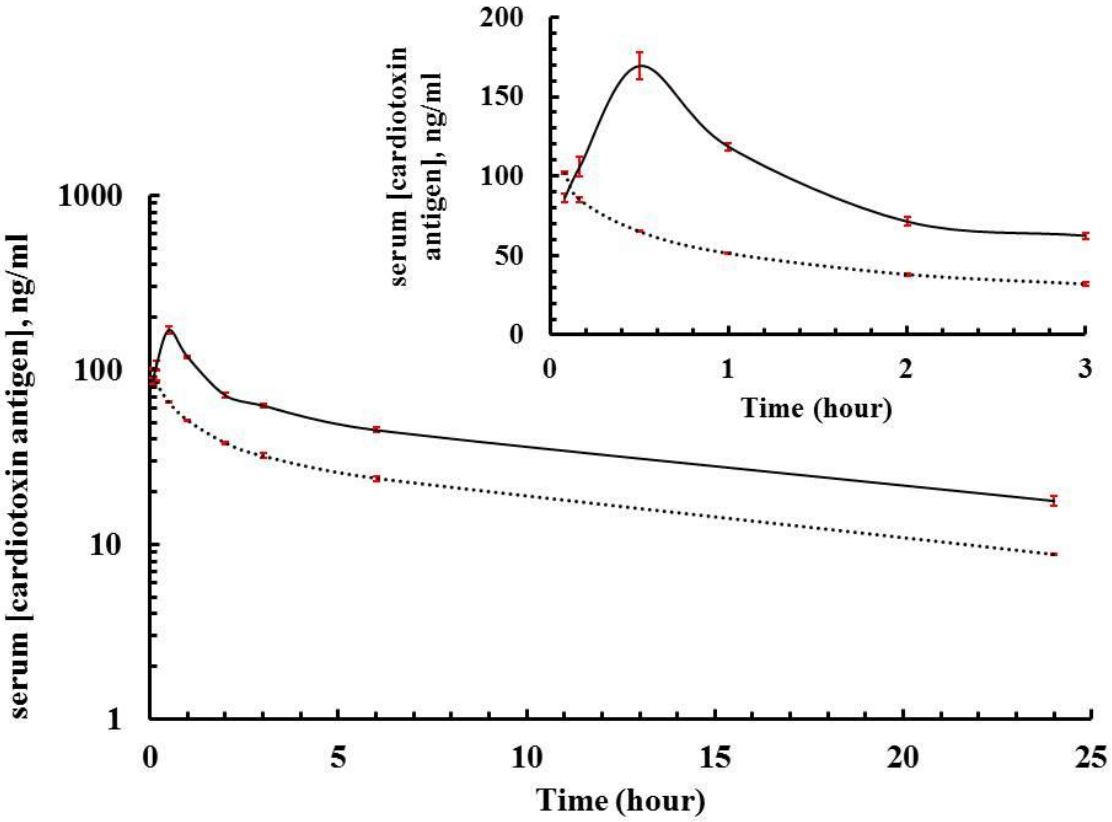


Figure 7.4: Serum concentration-time profile of cardiotoxin following intravenous and intramuscular injection of the whole *N. sumatrana* venom (in semi-logarithmic scale).

The serum cardiotoxin concentrations following intravenous (•••••••) and intramuscular (——) injection of the whole *N. sumatrana* venom were determined by double sandwich ELISA, and data are given as means ± S.D. (n = 3). The insert shows serum concentration-time profile of cardiotoxin following intramuscular injection of the whole venom (in arithmetic plot) during the first 3 h.

Table 7.5: Pharmacokinetics parameters of cardiotoxin following intravenous and intramuscular administration of whole *N. sumatrana* venom into rabbits.

Parameters	Intravenous		Intramuscular	
	Injection of whole venom	Injection of cardiotoxin	Injection of whole venom	Injection of cardiotoxin
A(ng/ml)	61.33 ± 1.24	63.61 ± 4.15	-	-
α (h^{-1})	1.43 ± 0.03	1.25 ± 0.20	-	-
B (ng/ml)	39.13 ± 1.15	41.36 ± 1.26	73.70 ± 3.02 (29.12 ± 1.19)[#]	59.87 ± 2.20 (27.27 ± 1.00)[#]
β (h^{-1})	0.063 ± 0.001	0.081 ± 0.0012	0.06 ± 0.004	0.085 ± 0.001
$T_{1/2\alpha}$ (h)	0.48 ± 0.01	0.56 ± 0.08	-	-
$T_{1/2\beta}$(h)	11.00 ± 0.17	8.59 ± 0.12	11.60 ± 0.87	8.15 ± 0.10
C_{max} (ng/ml)			169.35 ± 8.45 (66.91 ± 3.33) [#]	132.95 ± 5.69 (60.63 ± 2.59)[#]
k_{12} (h^{-1})	0.75 ± 0.01	0.60 ± 0.12	-	-
k_{21}(h^{-1})	0.60 ± 0.02	0.54 ± 0.07	-	-
$V_{d,area}$ (L)	1.90 ± 0.07	2.15 ± 0.05	2.04 ± 0.16	2.04 ± 0.03
V_c(L)	0.77 ± 0.01	0.95 ± 0.05	-	-
V_p(L)	0.99 ± 0.04	1.06 ± 0.02	-	-
CL (ml/h)	119.79 ± 2.35	173.73 ± 2.12	121.65 ± 0.18	173.71 ± 0.001
$AUC_{0-\infty}$ (ng/ml.h)	668.01 ± 12.96	575.67 ± 7.02	1320.13 ± 35.64 (264.03 ± 7.13)*	788.28 ± 23.91 (262.73 ± 7.97)*
Bioavailability, *F* (%)	100	100	39.5 ± 1.1	45.6 ± 0.1

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 7.4.

* $AUC_{0-\infty}$ values were adjusted to the dosage of *i.v.* injection, *i.e.* 0.1 mg/kg.

[#] B and C_{max} values were adjusted accordingly to $F_{i.m.}$ *i.e.* 39.5% (whole venom) and 45.6% (cardiotoxin).

7.4 DISCUSSION

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 central and peripheral compartments. The initial distribution half-life of *N. sumatrana* venom ($T_{1/2\alpha}$ = 0.77 h) following *i.v.* administration is comparable to the value obtained for *N. sputatrix* venom in the earlier study ($T_{1/2\alpha}$ = 0.46 h; Chapter 6) and to that for the African cobra venoms ($T_{1/2\alpha}$ = 22.2-30.5 min; Ismail *et al*., 1996) although a three compartment pharmacokinetic model was applied in the latter case. The terminal half-life ($T_{1/2\beta}$ = 13.64 h) obtained for the *i.v.* pharmacokinetics of *N. sumatrana* venom was not statistically significant different ($p > 0.05$) to that of *N. sputatrix* venom ($T_{1/2\beta}$ = 15.38 h; Chapter 6) when administered intravenously, indicating that the elimination of the *N. sumatrana* venom antigens occurred at the same rate as *N. sputatrix* venom antigens.

The large volume of distribution of *N. sumatrana* venom (1.79 L) which is more than 10 folds of the total blood volume of a rabbit (approximately 120-150 ml for a 2-kg rabbit), suggests that the venom antigens have substantially distributed to extravascular tissues. 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).

The volume of central compartment (0.99 L), comprising the blood and other highly perfused tissues, was comparable to the volume of peripheral compartment (0.77 L). The rate constant k_{12} refers to the fraction of venom antigens transferred in unit time from the central to the peripheral compartments and k_{21} is the rate constant for the reverse transfer process. The value of k_{12} is not significantly different ($p > 0.05$) from that of k_{21}, suggesting that the amount

of venom antigens in the central compartment is similar to that in the peripheral compartment at equilibrium, despite a large volume of distribution ($V_{d,area}$). This finding is in corroborate with the earlier pharmacokinetic study of *N. sputatrix* venom ($k_{12}/k_{21} = 1$; Chapter 6).

In an attempt to mimic the real pathophysiological situation in snake envenomation, the pharmacokinetics of *N. sumatrana* venom was studied following intramuscular administration of venom into rabbits. After the intramuscular of a sub-lethal dose of venom, the serum venom antigen levels increased immediately in the first 5 min before it declined, indicating relatively quick absorption of some venom antigens from the site of injection into the systemic circulation. However, the serum concentration-time profile of the intramuscularly injected whole venom yields apparently indistinguishable absorption and distribution phases of the various toxins, reflecting the continuous absorption of various antigenic venom components occurring at the same time as their respective distribution phase. There was a lag observed during the absorption phase probably due to absorption of venom via the lymphatic route from site of injection into blood circulation (Paniagua *et al*., 2012). There was only one major serum peak at 1 h, which was presumably caused by the merged absorption of a bulk of phospholipase A_2 (T_{max} ~ 2 h) and three-finger toxins (T_{max} ~ ½ h) into systemic circulation occur at the rate in close proximity with each other. Unlike that observed for the whole venom, there was no fluctuation pattern during absorption and/or distribution phase in the serum concentration-time profile of toxins. The significant differences in the pharmacokinetics of the whole venom and toxins were also reflected by the time to reach the peak concentration (T_{max}). Neurotoxin and cardiotoxin antigens reached their respective peak concentrations within 30 min, when compared to

phospholipase A_2, which reached its peak concentration within 2 h, reflecting fast absorption of neurotoxin and cardiotoxin from the site of injection to systemic circulation. The fast absorption of neurotoxin and cardiotoxin suggests the rapid onset of systemic symptoms upon cobra bites, which has been reported earlier (Paul, 1993 and Reid, 1964).

The terminal half-life ($T_{1/2\beta}$), volume of distribution by area ($V_{d,area}$) and the systemic clearance (CL) of the venom antigen following *i.m.* injection were not significantly different from that of *i.v.* routes ($p > 0.05$). This suggests that the terminal phase of the serum concentration-time profile for intramuscular route likely represents the elimination of venom antigens.

The terminal half-life ($T_{1/2\beta}$) and volume of distribution by area ($V_{d,area}$) obtained for *N. sumatrana* venom was not substantially different from the values obtained for *N. sputatrix* venom ($T_{1/2\beta}$ = 15.4 h and $V_{d,area}$ =1.5 L; Chapter 6). However, the systemic clearance (CL) of *N. sumatrana* venom following *i.v.* and *i.m.* administration was significantly higher than the systemic clearance of *N. sputatrix* venom (CL = 68.7 ml/h; Chapter 6), probably that some of the venom components in *N. sumatrana* venom were cleared from the systemic circulation in a faster manner. This is presumably due to a faster clearance of neurotoxin and cardiotoxin in *N. sumatrana* venom as demonstrated in the later pharmacokinetic studies of each individual toxin. The venom composition of *N. sumatrana* differs from *N. sputatrix* in the relative content of neurotoxin, cardiotoxin and phospholipase A_2. As demonstrated from ion exchange separation studies (Chapter 4), *N. sumatrana* venom consists of more neurotoxin and cardiotoxin (17% and 40%, respectively) compared to *N. sputatrix* venom (consists of 8% neurotoxin and 35% cardiotoxin). However, *N. sputatrix* venom

has substantially greater amount (35%) of phospholipase A_2 than *N. sumatrana* venom (28%).

The bioavailability of the *N. sumatrana* venom following *i.m.* injection was 41.9%, indicating an incomplete absorption of the antigenic venom components from the site of injection into systemic circulation. This may be due to the strong affinities of the cobra venom toxins at the site of injection (Guo *et al*., 1993 and Tseng *et al*., 1968). The bioavailability of the *N. sumatrana* venom ($F_{i.m.}$) was similar to that of the *N. sputatrix* venom ($F_{i.m.}$ = 41.7%), which is a reflection of the fact that cardiotoxin represents a bulk of both venoms and $F_{i.m.}$ of cardiotoxin was ~ 46%.

It is virtually impossible to investigate the pharmacokinetics of each individual toxin when whole venom was administered into experimentally envenomed rabbits. The whole venom is a reservoir of more than hundreds of proteins and peptides, and there are limitations for the quantitative measurements of individual toxins in envenomed animals' serum by the anti-toxin IgGs using ELISA assay. This is because of the immunological cross-reactivity observed among the snake venom toxins (Harrison *et al.,* 2003). The immunological cross-reactions studies revealed an extensive cross-reactivity between phospholipase A_2 and neurotoxin purified from *N. sumatrana* venom it suggests the possibility of unrelated proteins of distinctive primary structures and biological functions sharing common antigenic domains (Stábeli *et al*., 2005 and Tan *et al*., 1993).

As such, in the present report the pharmacokinetics of *N. sumatrana* venom purified phospholipase $A_{2,}$ neurotoxin and cardiotoxin was studied after intravenous and intramuscular injections of a sub-lethal dose of each individual

toxin into rabbits. Double-sandwich ELISA was developed in which specific anti-toxin IgG's (*i.e.* anti-phospholipase A_2 IgG, anti-neurotoxin IgG, anti-cardiotoxin IgG) were used to measure the serum toxin antigen levels following the *i.v.* and *i.m.* injections of the individual toxins into rabbits.

The serum concentration-time profiles of the toxins injected intravenously was best fitted to an open two-compartment pharmacokinetic model where the toxins were distributed between central and peripheral compartments. Following intravenous administration of individual toxins, phospholipase A_2, neurotoxin and cardiotoxin possessed a faster distribution half-life (0.56-0.66 h) compared to when whole venom was injected intravenously (0.93 h, $p < 0.05$), reflecting a more rapid distribution of the toxins upon entering into systemic circulation.

Furthermore, all the three toxins exhibited a large volume of distribution ($V_{d,area}$ = 1.60-2.16 L), which are >10 fold of the total blood volume of a rabbit, suggesting that the toxin antigens were extensively distributed to extravascular tissues. Both neurotoxin and cardiotoxin (2.04-2.16 L) had a larger $V_{d,area}$ compared to phospholipase A_2 and whole venom (1.39-1.79 L; $p < 0.05$). This is presumably that low molecular weight proteins such as neurotoxin and cardiotoxin (possess molecular weight of 8 and 9 kDa, respectively) move across the capillary wall more easily than larger proteins (Audebert *et al.*, 1994), such as phospholipase A_2 (16 kDa). The volume of peripheral compartment (V_p) was about 58% of the $V_{d,area}$, implying that the toxins displayed a stronger binding affinity to the tissues in the peripheral compartment.

In this study, phospholipase A_2 appeared to have a higher k_{12}/ k_{21} ratio (1.8) compared to neurotoxin (1.15) and cardiotoxin (1.1; $p < 0.05$), suggesting that at equilibrium, the amount of phospholipase A_2 antigens in peripheral compartment

is larger than in the central compartment, which indicates phospholipase A_2 exhibited a stronger affinity for target tissues to exert pharmacology activities such as myotoxicity, cytotoxicity, cardiotoxicity, hemolytic, anticoagulant activities and edema inducing activities (Bhat and Gowda, 1989; Das *et al.*, 2011; Fletcher *et al.*, 1982; Geoghegan *et al.*, 1999; Mukherjee, 2008; Yamaguchi, 2001 and Zhao *et al.*, 2000).

In this study, the terminal half-lives ($T_{1/2\beta}$) of neurotoxin and cardiotoxin were comparable to each other (8.59-8.75 h; *i.v.*), but both were significantly ($p < 0.05$) lower (faster elimination) than that of phospholipase A_2. However, the $T_{1/2\beta}$ values are substantially different from the terminal half-lives of African cobras' α-neurotoxin (15-29h; Ismail *et al.*, 1996) and that of cytotoxin from Chinese cobra, *Naja naja atra* (3.5-5.9h; Guo *et al.*, 1993).

From the $T_{1/2\beta}$ comparison, it can be expected that neurotoxin and cardiotoxin have significantly higher systemic clearance ($p < 0.05$) than phospholipase A_2 and whole venom, while there was no significant differences between the systemic clearance of phospholipase A_2 and whole venom. This implies that neurotoxin and cardiotoxin was eliminated from systemic circulation more quickly than phospholipase A_2 or the whole venom. Assuming that the elimination takes place primarily from the central compartment, the faster clearance of neurotoxin and cardiotoxin can be explained by the higher vascular permeability of the two toxins as both are low molecular weight proteins, result in a more rapid clinical recovery (Rojnuckarin, 2001).

It should be noted that neurotoxin had an intramuscular bioavailability ($F_{i.m.}$ = 81.5%) higher than phospholipase A_2 and cardiotoxin, indicates a more complete absorption of the neurotoxin from the site of injection. This is in agreement with

the finding of Ismail *et al.* (1998), who reported a bioavailability of 88% for *Walterinnesia aegyptia* α-neurotoxin. In contrast, $F_{i.m.}$ of cardiotoxin was only 45.6%, this was presumably due to the strong binding affinity of cardiotoxin with the tissues at the site of injection that probably resulted in a poor absorption of cardiotoxin into systemic circulation. Similar finding has been reported by Tseng *et al*. (1968), who found that only 30% of the injected *Naja naja atra* cardiotoxin was absorbed within 4 h. On the other hand, the $F_{i.m.}$ of the phospholipase A_2 was 68.6%, which was significantly higher than that of the whole venom (41.9%) The incomplete absorption of phospholipase A_2 and cardiotoxin may account for the local symptoms including necrosis, local inflammation and pain (Ownby *et al*., 1993 and Teixeira *et al*., 2003) upon envenomation by *N. sumatrana* venom.

Although the *i.v.* pharmacokinetic parameters of neurotoxin are similar to that of cardiotoxin, 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 cardiotoxins.

Due to the minimal immunological cross-reactivity observed 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 double-sandwich ELISA. This study would help to shed light on whether other venom components would significantly affect the pharmacokinetics of an individual toxin, and whether the information gathered from the study of pharmacokinetics of individual toxins can be applied when the whole venom was injected.

It is noted that when whole venom was injected, cardiotoxin exhibited a higher $T_{1/2\beta}$ and lower CL (both intravenous and intramuscular routes, $p < 0.05$) 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 mixture due to the competition among various venom components for the elimination processes. However, other than these two parameters, other pharmacokinetic parameters of cardiotoxin when injected alone are comparable to those when whole venom was injected. These results seem to suggest that information gathered from the studies of the pharmacokinetics of the individual main toxins when each toxin was administered individually can be largely applied to the situation when the whole venom was injected, with the possible exception that the rate of elimination of the toxins determined this may be higher than when whole venom was injected.