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COMPARISON OF THE *IN VITRO* NEUROMUSCULAR ACTIVITY OF VENOM FROM THREE AUSTRALIAN SNAKES (*HOPLOCEPHALUS STEPHENSI*, *AUSTRELAPS SUPERBUS* AND *NOTECHIS SCUTATUS*): EFFICACY OF TIGER SNAKE ANTIVENOM

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SUMMARY

1. Tiger snake antivenom, raised against *Notechis scutatus* venom, is indicated not only for the treatment of envenomation by this snake, but also that of the copperhead (*Austrelaps superbus*) and Stephen's banded snake (*Hoplocephalus stephensi*). The present study compared the neuromuscular pharmacology of venom from these snakes and the *in vitro* efficacy of tiger snake antivenom.

2. In chick biventer cervicis muscle and mouse phrenic nerve diaphragm preparations, all venoms (3–10 μg/mL) produced inhibition of indirect twitches. In the biventer muscle, venoms (10 μg/mL) inhibited responses to acetylcholine (1 mmol/L) and carbachol (20 μmol/L), but not KCl (40 mmol/L). The prior (10 min) administration of 1 unit/mL antivenom markedly attenuated the neurotoxic effects of *A. superbus* and *N. scutatus* venoms (10 μg/mL), but was less effective against *H. stephensi* venom (10 μg/mL); 5 units/mL antivenom attenuated the neurotoxic activity of all venoms.

3. Administration of 5 units/mL antivenom at t_{90} partially reversed, over a period of 3 h, the inhibition of twitches produced by *N. scutatus* (10 μg/mL; 41% recovery), *A. superbus* (10 μg/mL; 25% recovery) and *H. stephensi* (10 μg/mL; 50% recovery) venoms. All venoms (10–100 μg/mL) also displayed signs of *in vitro* myotoxicity.

4. The results of the present study indicate that all three venoms contain neurotoxic activity that is effectively attenuated by tiger snake antivenom.

Key words: antivenom, copperhead, myotoxicity, neuromuscular junction, neurotoxicity, Stephen's banded snake, tiger snake, venom.

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INTRODUCTION

Australia is home to a collection of the world's most venomous elapid snakes. Based on the clinical effects of envenomation and the type of monovalent antivenom required for treatment, it has been proposed that these snakes may be classified into five groups.[1] One such group includes tiger snakes, copperheads and banded/broad-headed snakes because Commonwealth Serum Laboratories (CSL) tiger snake antivenom is indicated for the treatment of systemic envenomation by all these snakes. In fact, it has been suggested that the common (lowland) copperhead (*Austrelaps superbus*) is so closely related to the common tiger snake (*Notechis scutatus*) that it should be included in the *Notechis* genus.[2] In addition, tiger snake antivenom has been used to reverse coagulation anomalies in patients after envenomation by Stephen's banded snake (*Hoplocephalus stephensi*).[3]

Although a considerable amount of research has been directed at the neurotoxic components of the venom from *N. scutatus*,[4–10] the venom of *A. superbus* has been understudied, except for the identification of some anticoagulant and antiplatelet activity.[11–13] While the venom of *H. stephensi* has been shown to display haemorrhagic and procoagulant activities,[14,15] any neurotoxic components of this venom have been largely ignored. The present study compared the neuromuscular pharmacology of venom from these snakes and the efficacy of tiger snake antivenom against the *in vitro* neurotoxicity. In addition, the venoms were profiled using on-line liquid chromatography–mass spectrometry (LC-MS) to examine variations in venom composition.

METHODS

Chick biventer cervicis nerve–muscle preparation

Chicks (4–10 days old) were killed by CO_2 and exsanguination and the biventer cervicis muscles were removed and mounted in tissue baths (10 mL) under a resting tension of 1 g. Tissues were bathed in modified physiological salt solution (34°C), bubbled with 95% O_2 and 5% CO_2, of the following composition (in mmol/L): NaCl 118.4; $NaHCO_3$ 25; glucose 11; KCl 4.7; $MgSO_4$ 1.2; KH_2PO_4 1.2; $CaCl_2$ 2.5. Twitches were evoked by stimulating the motor nerve at a voltage greater than that required to evoke a maximal twitch (0.1 Hz, 0.2 msec). In the absence of nerve stimulation, submaximal responses to acetylcholine (ACh; 1 mmol/L, 30 s), carbachol (CCh; 20 μmol/L, 60 s) and KCl (40 mmol/L, 30 s) were obtained before and after the addition of venom.[10]

For direct muscle stimulation (0.1 Hz, 2 msec and a voltage greater than that required to evoke a maximal twitch), neuromuscular transmission was abolished by the continuous presence of tubocurarine (10 μmol/L) and the electrodes placed around the belly of the muscle.

Mouse phrenic nerve–diaphragm preparation

Hemidiaphragms were dissected from male mice (20–25 g) and mounted in 10 mL organ baths containing physiological salt solution (as described above; 37°C). The attached phrenic nerve was stimulated at a voltage greater than that required to evoke a maximal twitch (0.1 Hz, 0.2 msec).

Antivenom experiments

Where indicated, tiger snake antivenom (1 or 5 units/mL) was added 10 min prior[17,17] to the addition of venoms (10 μg/mL). Additional experiments were undertaken to examine the reversibility of the inhibitory effects of the venoms. In these experiments, tiger snake antivenom (5 units/mL) was added after the venoms had produced a 90% inhibition of nerve mediated twitches (i.e. at t_{90}).[17] Twitch height was then monitored for a further 3 h period.

Liquid chromatography–mass spectroscopy analysis

On-line LC-MS of venom samples dissolved in 0.1% trifluoroacetic acid (TFA) to a concentration of approximately 1 mg/mL was performed on a Vydac C18 analytical column (2.1 × 250 mm, 5 μm particle size, 300 Å; Alltech Associates, Sydney, NSW, Australia) with solvent A (0.05% TFA) and solvent B (90% acetonitrile in 0.045% TFA) at a flow rate of 130 μL/min. The solvent delivery and gradient formation of 0–90% acetonitrile/0.05% TFA over 90 min was achieved using an Applied Biosystems 140B solvent delivery system (Applied Biosystems, Scoresby, Victoria, Australia). Electrospray mass spectra were acquired on a PE-SCIEX III triple quadrupole mass spectrometer (MDS Sciex, Ontario, Canada) equipped with an ionspray ionization source. The electrospray Sprayer voltage was 5200 V, Orifice 120 V, Ring 280 V and the Skimmer 80 V. Samples (10 μL) were injected manually into the LC-MS system and analysed in positive ion mode. Full scan data were acquired over the mass range 400–2100 Da with a step size of 0.2 amu. Data processing was performed with the aid of the software package Biomultiview (PE-SCIEX, Ontario, Canada).

Drugs

Acetylcholine chloride, carbamylcholine chloride (carbachol) and D-tubocurarine chloride were purchased from Sigma (St Louis, MO, USA). Freeze-dried venoms were purchased from Venom Supplies (Tanunda, SA, Australia). Pooled venoms from different snakes were used for all species to minimize the effects of individual variations. The same batch of venom was used for all studies. Stock solutions were prepared by dissolving the venoms in 0.1% bovine serum albumin (BSA) in saline (0.9% w/v). These were aliquoted and stored at –20°C until required.

Statistics

Data were analysed using GraphPad Prism (GraphPad Software, San Diego, CA, USA) and SigmaStat (SPSS Science, Chicago, IL, USA). The t_{90} values were compared by two-way ANOVA. Comparison of experiments where antivenom was added at t_{90} was undertaken using a two-way repeated-measures ANOVA to compare differences between the three venoms at all time points. Post hoc pairwise multiple comparisons were performed using Bonferroni's method. Statistical significance was indicated when $P < 0.05$. All data are expressed as the mean±SEM.

RESULTS

Effect of venoms on indirect twitches

Chick biventer cervicis preparation

All three venoms at 3 μg/mL (Fig. 1a) and 10 μg/mL (Fig. 1b) produced time-dependent block of indirect twitches in the chick biventer cervicis preparation with the following rank order of potency: *N. scutatus* ≥ *H. stephensi* > *A. superbus* (Fig. 1; Table 1). There was a significant difference between the t_{90} values for

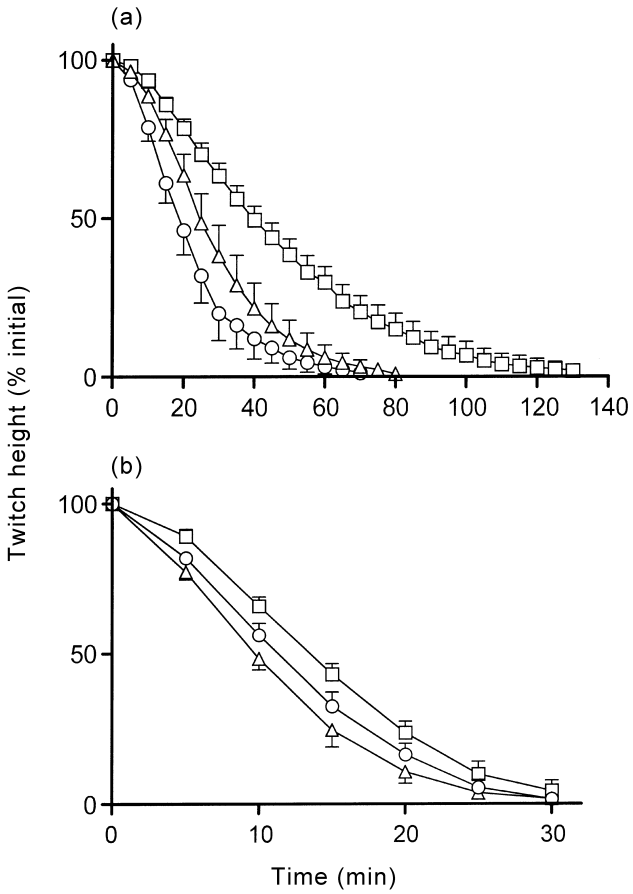

Fig. 1 Effect of *Notechis scutatus* (○), *Hoplocephalus stephensi* (△) and *Austrelaps superbus* (□) venoms at (a) 3 μg/mL (n = 7–8) and (b) 10 μg/mL (n = 11–12) on indirect twitches of the chick isolated biventer cervicis nerve–muscle preparation.

Table 1 Time (min) taken to cause 90% inhibition of indirect twitches

	Chick biventer cervicis		Mouse diaphragm	
	3 μg/mL	10 μg/mL	3 μg/mL	10 μg/mL
Notechis scutatus	38 ±6 (8)	22 ±2 (12)	29 ±4 (9)	21 ±3 (5)
Hoplocephalus stephensi	47 ±7 (8)	20 ±2* (11)	75 ±8† (5)	25 ±4* (5)
Austrelaps superbus	89 ±10† (7)	26 ±3* (11)	110 ±8† (7)	45 ±7*† (5)

Data are the mean ±SEM with the number of experiments given in parentheses. *$P < 0.05$ compared with 3 μg/mL of the corresponding venom (two-way ANOVA); $^{\dagger}P < 0.05$ compared with *N. scutatus* venom at the corresponding concentration (two-way ANOVA).

H. stephensi and *A. superbus* venoms at a concentration of 3 μg/mL compared with 10 μg/mL ($P < 0.05$; Table 1), indicative of a concentration-dependent inhibitory effect. However, there was no significant difference between the t_{90} values at concentrations of 3 and 10 μg/mL for *N. scutatus* venom, indicating a near maximal effect at 3 μg/mL in this preparation.

All three venoms (10 μg/mL) abolished contractile responses to exogenous ACh (1 mmol/L) and CCh (20 μmol/L), but responses to KCl (40 mmol/L) were not significantly inhibited (i.e. 90 ± 13, 87 ± 4 and 100 ± 8% of initial response for *N. scutatus*, *H. stephensi* and *A. superbus*, respectively; $P > 0.05$ compared with time control).

Mouse diaphragm

All three venoms at 3 μg/mL (Fig. 2a) and 10 μg/mL (Fig. 2b) produced time-dependent block of indirect twitches in the mouse diaphragm preparation with the same rank order of potency displayed in the chick biventer (i.e. *N. scutatus* ≥ *H. stephensi* > *A. superbus*; Fig. 2; Table 1).

There was a significant difference between the t_{90} values for *H. stephensi* and *A. superbus* venoms at a concentration of 3 μg/mL compared with 10 μg/mL ($P < 0.05$; Table 1), indicative of a concentration-dependent inhibitory effect. However, there was no significant difference between the t_{90} values at concentrations of 3 and 10 μg/mL for *N. scutatus* venom, indicating a near maximal effect at 3 μg/mL in this preparation.

Efficacy of tiger snake antivenom

Pre-incubation of antivenom

Prior incubation (10 min) with 1 unit/mL tiger snake antivenom in the chick biventer cervicis preparation markedly attenuated the inhibitory effects of *N. scutatus* and *A. superbus* venoms (10 μg/mL), but was relatively ineffective against the inhibitory effects of *H. stephensi* venom (10 μg/mL; Fig. 3a). However, prior incubation with 5 units/mL tiger snake antivenom abolished the inhibitory effects of all three venoms (10 μg/mL; Fig. 3a).

Antivenom added at t_{90}

The addition of tiger snake antivenom (5 units/mL) at t_{90} significantly reversed, over a period of 3 h, the inhibition of twitches produced by 10 μg/mL of all three venoms (i.e. 41 ± 6, 50 ± 4 and 25 ± 2% recovery for *N. scutatus*, *H. stephensi and A. superbus*, respectively; Fig. 3b). There was no spontaneous recovery of

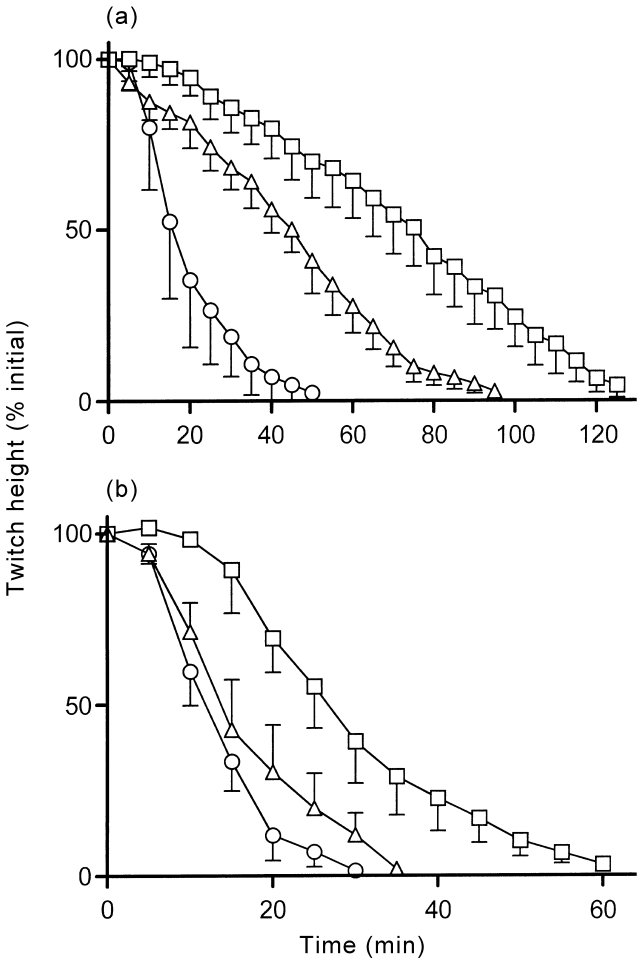

Fig. 2 Effect of *Notechis scutatus* (○), *Hoplocephalus stephensi* (△) and *Austrelaps superbus* (□) venoms at (a) 3 μg/mL ($n = 4–7$) and (b) 10 μg/mL ($n = 5$) on indirect twitches of the mouse phrenic nerve–diaphragm preparation.

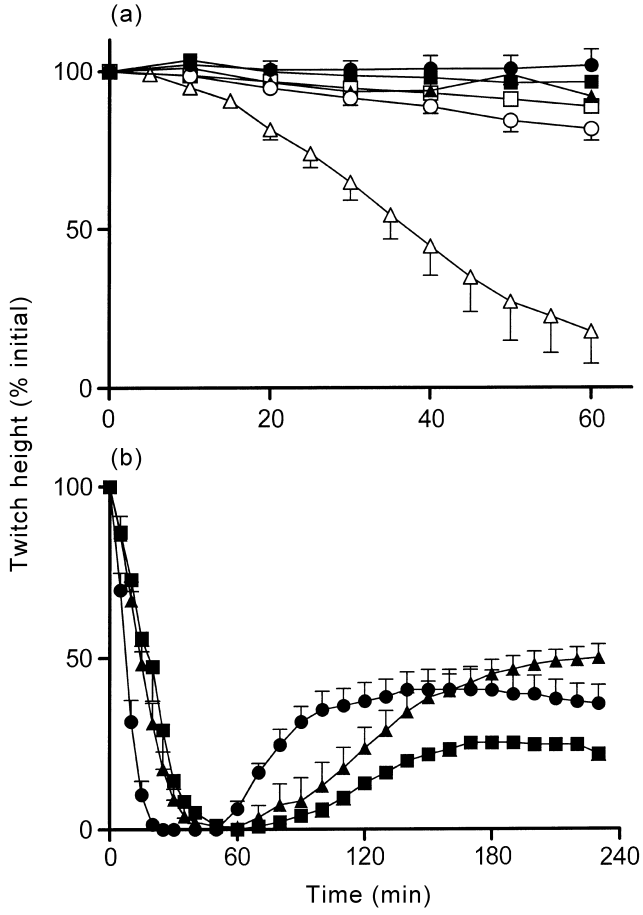

Fig. 3 Effect of tiger snake antivenom (1 unit/mL, open symbols; 5 units/mL, closed symbols) added (a) 10 min prior to *Notechis scutatus* (○; ●), *Hoplocephalus stephensi* (△; ▲) and *Austrelaps superbus* (□; ■) venoms (10 μg/mL; $n = 4–6$) or (b) after twitch height had been reduced by 90% (i.e. at t_{90}; $n = 5–6$) on indirect twitches of the chick isolated biventer cervicis nerve–muscle preparation.

twitches when venoms were left in contact with the preparation, in the absence of antivenom, for a period of 3 h (n = 4–6; data not shown).

Effect of venoms on direct twitches

Austrelaps superbus and *N. scutatus* venoms (10 μg/mL) produced significant inhibition of direct twitches in the chick biventer cervicis preparation, indicative of myotoxicity (Fig. 4a). However, *H. stephensi* venom had no significant effect at this concentration (Fig. 4a). At a higher concentration (i.e. 100 μg/mL), all three venoms produced marked inhibition of direct twitches (Fig. 4b). In addition, all three venoms produced a slowly developing contractile response in this preparation (data not shown).

Liquid chromatography–mass spectrometry analysis of venoms

Venoms were profiled using LC-MS to determine differences in venom composition (Fig. 5). All venoms were found to contain a number of components with molecular weights corresponding to post-synaptic (6–8 kDa) and presynaptic (12–14 kDa) neurotoxins.[18] A single peak with a molecular weight of 46 kDa (consistent with a subunit of a prothrombin activator[16]) was evident in the *N. scutatus* and *H. stephensi* venoms, but not in the *A. superbus* venom. Evident in all three venoms were a number of compounds with molecular weights of 3–4 kDa, consistent with that of natriuretic peptides.[19] Also present in the *H. stephensi* and *N. scutatus* venoms were 23 kDa components with retention times consistent with cyclic nucleotide-gated ion channel-blocking toxins.[20] The venoms displayed only minimal homology to each other in regards to possessing compounds with the exact same retention time and molecular weight.

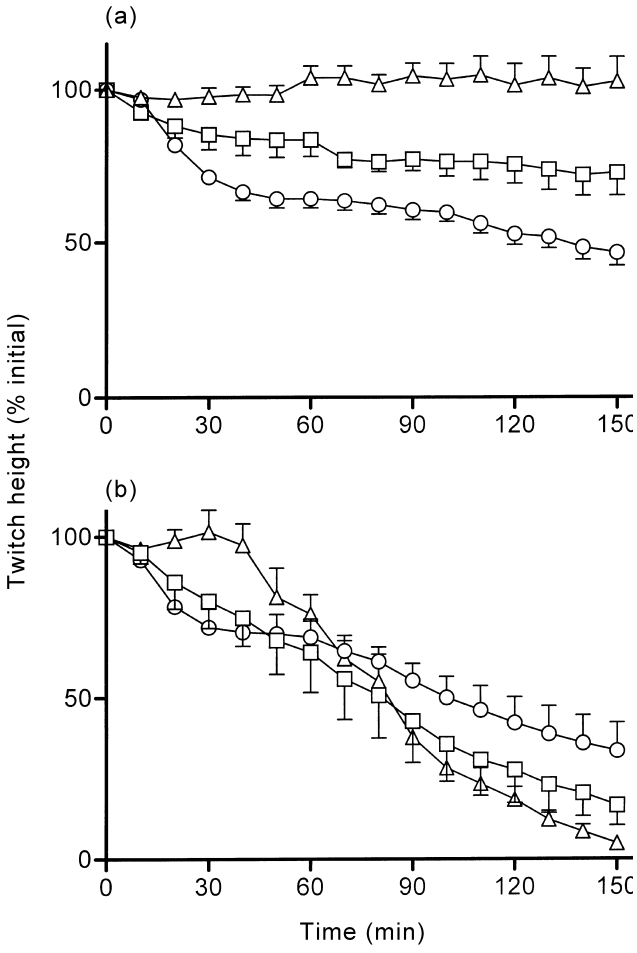

Fig. 4 Effect of *Notechis scutatus* (○), *Hoplocephalus stephensi* (△) and *Austrelaps superbus* (□) venoms at (a) 10 μg/mL (n = 5–6) and (b) 100 μg/mL (n = 4–6) on direct twitches of the chick isolated biventer cervicis nerve–muscle preparation.

DISCUSSION

While there is little doubt that systemic envenomation by *N. scutatus* is associated with marked neurotoxicity, there is considerably less known regarding the nature of *H. stephensi* envenomation.[21] Unlike *N. scutatus*, *H. stephensi* are semi-arboreal and have a narrower range of distribution,[22] perhaps contributing to the low number of documented cases of envenomation by this species. Two recent reports on the clinical effects of envenomation by *Hoplocephalus* species documented rapidly developing severe hypotension and coagulation disturbances. No signs of neurotoxicity were evident.[3] The limited experimental studies performed on *H. stephensi* crude venom have focused on the presence of haemorrhagic and procoagulant activities,[14] as well as the lethality of the venom in whole animal studies.[23]

It has been suggested that members of the genus *Austrelaps* rarely bite humans and that considerable provocation is required before they will strike in anger.[22] However, the effects of envenomation can be severe. One report detailed profound paralysis in a dog following a bite by this species.[22] Because the murine subcutaneous LD_{50} of this species is 0.5 mg/kg[24] and the venom yield is 26–85 mg,[22] this species should be considered capable of a lethal envenomation.

The results of the present study indicate that all three venoms studied contain post-synaptic neurotoxic activity, as evidenced by their inhibition of indirect twitches in both neuromuscular preparations and their selective inhibition of exogenous nicotinic receptor agonists. Although not consistent with the clinical effects of envenomation by some of the species, in particular *H. stephensi*, these results are supported by the LC-MS analysis of the venoms revealing components with molecular weights within the range of peptidic neurotoxins. These results enable us to make a comparison with the neurotoxic activity of venoms from some other Australian elapids that we have previously examined.[16,17,24,25] The three venoms used in the present study were less potent, as determined by t_{90} values (mean ± SEM, in minutes; all obtained at a venom concentration of 10 μg/mL), in the chick biventer cervicis preparation than *Acanthophis antarcticus* (14 ± 1) and *Acanthophis pyrrhus* (14 ± 1), equipotent with *Acanthophis praelongus* (19 ± 2) and *Oxyuranus microlepidotus* (27 ± 3), but considerably more potent than *Oxyuranus scutellatus* (42 ± 3) and *Oxyuranus scutellatus canni* (48 ± 5).

Interestingly, venom from the Papuan taipan (*O. s. canni*) was markedly more potent in the murine preparation (25 ± 1) compared with the avian preparation, whereas *Acanthophis* sp. venoms[18] and, in the present study, *N. scutatus* and *H. stephensi* venoms were approximately equipotent. Only the venom of *A. superbus* was less potent in the murine preparation. The reasons for these differences in sensitivity are unclear, but may be related to adaptive changes in

venom composition due to prey preference.[26] However, the dietary preferences of these snakes suggest that this is unlikely: *A. superbus*, reptiles (63%) and frogs (35%); *N. scutatus*, frogs (92%) and mammals (4%); *H. stephensi*, mammals (45%), reptiles (44%) and frogs (11%).[27]

In addition, the rank order of neurotoxicity of the venoms in the mouse diaphragm preparation (i.e. *N. scutatus* ≥ *H. stephensi* > *A. superbus*) is different than that reported for LD_{50} values in whole mice (s.c. in 0.1% BSA), where there were marked differences: *N. scutatus* (0.118 mg/kg) > *A. superbus* (0.5 mg/kg) > *H. stephensi* (1.44 mg/kg).[23] However, we have previously alluded to the limits of comparing these different techniques.[17]

All venoms displayed myotoxicity *in vitro* as evidenced by their inhibitory effects on direct twitches and slowly developing contractile response in the chick biventer cervicis.[10] *Hoplocephalus stephensi* venom displayed the weakest myotoxicity, having no

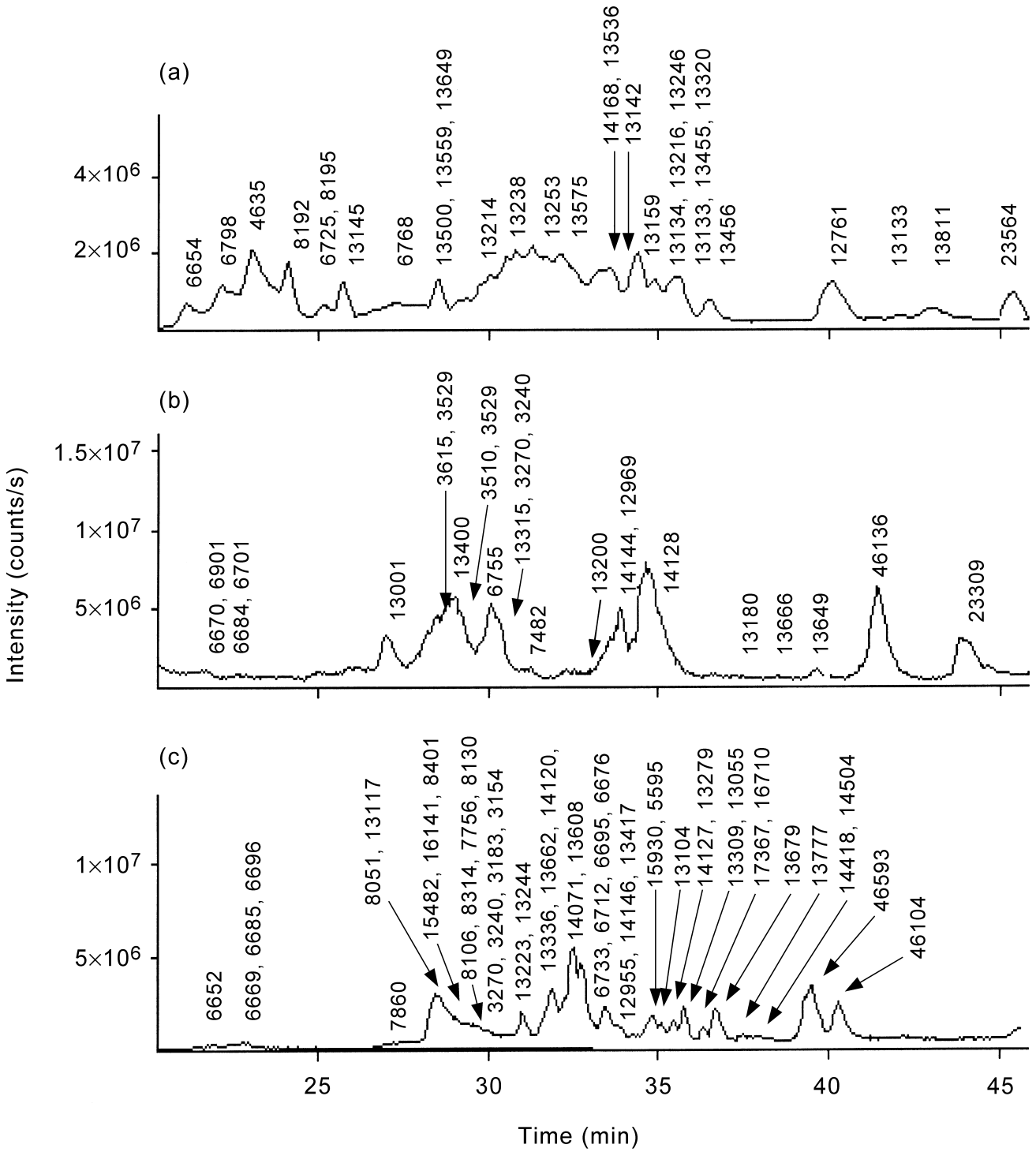

Fig. 5 Liquid chromatography–mass spectrometry profiles of (a) *Austrelaps superbus*, (b) *Hoplocephalus stephensi* and (c) *Notechis scutatus* venoms. Reconstructed masses are given above each peak. Intensity (counts/s) values are plotted on the *y*-axis.[1]

apparent effect at 10 μg/mL and a slower onset of activity at 100 μg/mL.

Despite the availability of a range of monovalent snake antivenoms in Australia targeted against a number of species (e.g. tiger snakes, taipans, death adders), quite a few snakes are not specifically covered. In these cases, antivenoms raised against other species are used. Therefore, tiger snake antivenom is recommended for envenomation by all species of *Austrelaps* and *Hoplocephalus*.[18] The results of the present study with tiger snake antivenom, either added prior to the venoms or after the venoms had produced considerable neuromuscular blockade, clearly show that the antivenom is effective against the *in vitro* neurotoxicity produced by all three venoms, supporting the current treatment recommendations. Tiger snake antivenom has been used previously to treat the coagulation abnormalities observed following *H. stephensi* envenomations.[3] Interestingly, in the present study, *H. stephensi* venom was relatively unaffected by the lower concentration of antivenom (i.e. 1 unit/mL added prior to venom), whereas the effects of *N. scutatus* and *A. superbus* were almost completely abolished. This may reflect differences in venom composition or in the quantities of individual components, which are neutralized by the antivenom, within the venoms.

This study provides further data regarding the potential effects of Australian elapids. While neurotoxicity has not been reported as a clinical feature of envenomation by *H. stephensi*, central to the clinical picture is profound hypotension followed by severe disruption of blood chemistry. These effects may be accounted for by the presence of putative natriuretic peptides and a prothrombin-activating enzyme[16] in the venom. Further work to isolate natriuretic peptides from this venom is underway. However, the current results indicate that neurotoxicity is potentially a complication of envenomation and that tiger snake antivenom may have a role in reversing these effects.

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