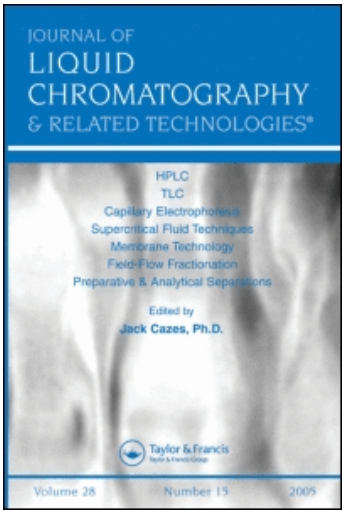

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Halassy, Beata , Brgles, Marija , Habjanec, Lidija , Balija, Maja Lang , Tomašić, Jelka , Križaj, Igor , Štrancar, Aleš and Barut, Miloš(2008) 'Use of Convective Interaction Media for Analysis of Long-Nosed Viper Venom', Journal of Liquid Chromatography & Related Technologies, 31: 1, 38 — 53

To link to this Article: DOI: 10.1080/10826070701665535

URL: http://dx.doi.org/10.1080/10826070701665535

Journal of Liquid Chromatography & Related Technologies®, 31: 38–53, 2008

ISSN 1082-6076 print/1520-572X online
DOI: 10.1080/10826070701665535

Use of Convective Interaction Media for Analysis of Long-Nosed Viper Venom

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Abstract: It was confirmed in this study that *Vipera ammodytes ammodytes* venom samples with a higher content of ammodytoxins (Atxs), basic neurotoxic phospholipases A_2, are more lethal, exposing Atxs as one of the major toxic components in the long-nosed viper venom. In addition, we recently correlated the ability of the venom to produce highly protective antiserum in rabbits with the amount of Atxs in the venom. Here, we developed a rapid, robust, and highly reproducible chromatographic method, based on HPLC separation on Convective Interaction Media to assess the quantity of Atxs in a particular venom sample in order to predict its potential to induce highly protective antiserum in immunized animals.

Keywords: *Vipera ammodytes ammodytes*, Ammodytoxins, Convective Interaction Media, Antivenom production, Long-nosed viper

INTRODUCTION

The venom of the long-nosed viper (*Vipera ammodytes ammodytes*), the most poisonous European snake, like the venom of any other snake, is a complex mixture of proteins and peptides, as well as non-protein material, with diverse pharmacological activities. Some venom components are not toxic, but some are highly toxic and are responsible for serious envenomation effects. The venoms have been used since 1894 for immunization of

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sheep and horses to produce specific antivenoms (venom specific antisera), that are still the only specific therapy for the victims of snake bites. Antivenoms contain antibodies against numerous venom components, irrespective of their toxicity. Several toxic compounds from the long-nosed viper venom have been purified and biochemically characterized so far.[1–4] The contribution of separate components of the venom to induction of toxicity neutralizing antibodies has, however, not been studied. Among the most interesting venom proteins in this respect are secreted phospholipases A_2 ($sPLA_2$s). They are abundantly present in the venom and express several pharmacological activities. Lethality wise, the most interesting $sPLA_2$s are presynaptically neurotoxic ammodytoxins (Atxs). Three isoforms have been found in the venom, AtxA, AtxB, and AtxC, which differ from each other in only 2 to 5 amino acid residues out of 122.[5] AtxA has been shown to also possess anticoagulant activity.[6] Myotoxicity of the venom is due to ammodytin L (AtnL), a structural analogue of $sPLA_2$.[7] While all of the pharmacologically active $sPLA_2$ molecules are basic proteins, the two non-toxic $sPLA_2$s that were found in the venom, ammodytins I_1 and I_2 ($AtnI_1$ and $AtnI_2$), are neutral proteins.[8] We have recently found that the protective efficacy of antisera from venom immunized rabbits highly correlated with the level of IgGs specific for AtxAs (Halassy et al., in preparation). The information about the content of Atx in the venom before using it for immunization might be, therefore, an important parameter for efficient antiserum production. The aim of this study was, therefore, to develop the method for fast extraction and partial separation of the basic venom components (among which are toxic $sPLA_2$s), to identify the basic PLA_2-containing fractions and to obtain the tool for studying the relationship between the venom composition and its ability to induce anti-AtxA antibodies in immunized rabbits, that so far seems to be relevant for antivenom efficacy.

In recent years, convective interaction media (CIM) disk monolithic columns have been shown as an excellent chromatographic support for fast and accurate separation and/or concentration of large biomolecules such as proteins, nucleic acids, and viruses.[9–12] In this paper, we report on development of a novel method for the separation and partial characterization of basic venom components from *V. a. ammodytes* venom using CIM CM (carboxymethyl) disk and HPLC. Using the new chromatographic procedure, we succeeded to separate basic components of *V. a. ammodytes* venom in a single run and assess their content. The new method was tested by comparing two batches of *V. a. ammodytes* venom with different potentials to induce high quality antiserum in experimental rabbits. We demonstrated that the content of basic Atxs in both venom samples was proportional to their lethality in mice and their anti-Atxs antibody producing power. Linked to our recent observation that the content of Atxs in the venom is linked to production of highly protective antisera in rabbits (Halassy, in preparation), the presented method might be used to rapidly

estimate the potential of particular venom sample for production of high quality antiserum.

EXPERIMENTAL

Reagents, Chemicals, and Instruments

Ultra-pure water was obtained from a Simplicity 185 System (Millipore, USA). Bovine serum albumin (BSA), Tween 20, Tris(hydroxymethyl)aminomethane (Tris) and *o*-phenylenediamine dihydrochloride (OPD) were from Sigma, USA. Horseradish peroxidase conjugated goat anti-rabbit IgG (HRP-anti-rabbit IgG) was from Bio-Rad Laboratories, USA. Chemicals for buffers and solutions were from Kemika, Croatia, unless stated otherwise. Microplate reader Reader 530 and microplate washer Multiwash were from Organon Teknika, Netherlands. Elution fractions were concentrated using Centricon YM3 (Millipore, USA). SDS polyacrylamid electrophoresis (SDS-PAGE) was carried out on a PhastGel System equipped with a development unit (Pharmacia, Sweden). Gradient gels, 8–25%, (GE Healthcare BioSciences AB, Sweden) were used and proteins were detected with silver. Low molecular weight markers were from GE Healthcare.

Chromatographic Media and Instrumentation

CIM CM (carboxymethyl, weak cation exchanger) disk monolithic column from BIA Separations (Ljubljana, Slovenia) was used. Disk bed volume was 0.34 mL, porosity 62%.

All chromatographic experiments were performed using Waters HPLC System: Waters 600 System Controller, Waters 600 Pump, Waters In-line Degasser AF, 2996 photodiode array detection (DAD) system, Empower Software (Waters, USA). Samples were applied to the disk using a 100 μL loop. Binding buffer was 50 mM Tris/HCl, pH 9.0. Elution buffer was 1 M NaCl in Tris/HCl buffer. Flow rate used throughout was 3 mL/min.

Snake Venoms and Venom Components

Two batches of snake venom named KR95 and SL05 (snakes collected from Croatian regions around Krapina in 1995 and from region around Slunj in 2005) were produced at Institute of Immunology, Croatia. AtxA, AtxB, AtxC, AtnL, and $AtnI_2$ were isolated from the long-nosed viper venom.[8,13] The recombinant AtxA was produced as described.[14] The homogeneity of PLA_2s was confirmed by the N-terminal amino acid sequencing and/or mass spectrometry. *Vipera berus* venom (VB) collected from the Novosibirsk area was obtained from Russia.

Specific Antisera

Polyclonal antisera obtained by immunization of rabbits (5 per group) with the same quantity of either KR95 or SL05, and using the same immunization procedure (3 immunizations in 28 day intervals, using ISA206^{PGM} experimental adjuvant[15]) were prepared at the Institute of Immunology and were named anti-KR95 and anti-SL05, respectively. Polyclonal antisera specific for AtxA (anti-AtxA) and $AtnI_2$ (anti-$AtnI_2$) were prepared as above, only the antigen for immunization was pure AtxA or $AtnI_2$, respectively.

Assay of Lethal Toxicity

The lethal toxicity of venoms, expressed as median lethal dose (LD_{50}), i.e., the amount (in μg) of dry venom causing the death in half of the mice population used was determined according to the method of Theakston and Reid[16] and European Pharmacopoeia 4th ed. (2002), with experimental details described by Lang Balija et al.[17] Toxicity of chromatographic fractions was assayed by the same procedure, only protein concentrations were adjusted to 5 μg/mice and 20 μg/mice by diluting the fractions with saline, and filtering them through 0.20 μm. The group of four mice was i.v. injected with the fraction to be tested, and the deaths were monitored 48 hours later.

ELISA for Detection of AtxA and $AtnI_2$-Containing Chromatographic Peak(s)

ELISA plates (Costar, USA) were coated with protein material from each chromatographic fraction (details in Results), diluted to a concentration of 1 μg/mL in carbonate buffer pH 9.6 (100 μL/well), and incubated overnight at 4°C. Also, positive control wells were coated with 1 μg/mL of pure AtxA and $AtnI_2$ solutions in carbonate buffer. After three washings with PBS/T buffer (0.05% (v/v) Tween 20 in PBS), the blocking step was done with 0.5% (w/v) BSA in PBS/T buffer (200 μL/well) for 2 h at 37°C. Plates were washed and anti-AtxA or anti-$AtnI_2$ were added in duplicates in serial two-fold dilutions and incubated overnight at RT. After washing HRP-anti-rabbit IgG (10,000 × diluted) was added (100 μL/well) and incubated 2 h at 37°C. Plates were washed and the OPD solution (0.6 mg/mL in citrate-phosphate buffer pH 5.0) was added (100 μL/well) and incubated for 30 min at RT in the dark. The enzymatic reaction was stopped with 1 M H_2SO_4 (50 μL/well) and absorbance at 492 nm (A492) was measured.

ELISA for Detection of Anti-AtxA IgG in Immunized Rabbits Sera

Anti-AtxA IgG was determined by ELISA as described above and modified as follows. Briefly, plates were coated with 1 μg/mL of pure AtxA solution in

carbonate buffer (100 μL/well) and left overnight at 4°C. After blocking with 0.5% BSA in PBS/T (200 μL/well) for 2 h at 37°C, rabbit sera to be tested (100 μL/well) and the "in house standard" were added in five binary dilutions in duplicates and incubated overnight at RT. Plates were washed, HRP-anti-rabbit IgG (100 μL/well) was added (10,000 × diluted), and incubated for 2 h at 37°C. After washing, the OPD solution (100 μL/well) was added and incubated for 30 min at RT in the dark. The enzymatic reaction was stopped with 1 M H_2SO_4 and A492 was measured. Quantitative determination of anti-AtxA IgG was done by parallel line assay,[15] comparing each serum to the "in house standard". Polyclonal serum that contained high levels of IgG specific for venom served as "in house standard" for determination of anti-AtxA IgG to which we voluntary assigned 10,000 arbitrary units per mL (AU/ml).

Statistics

The statistical significance of differences was assessed by Mann-Whitney U-nonparametric test using Statistica 6.0 for Windows, StatSoft, Inc.

RESULTS

HPLC of the Venom Using CIM CM Disk. Establishing Experimental Parameters

The conditions for separation of different basic components in snake venom were chosen as follows: 10 mg of venom (in 100 μL of Tris/HCl, pH 9.0) was applied to the disk during 9 minutes and the bound material was eluted with 0.04 M (Elution fraction 1, EF1), 0.08 M (EF2), 0.12 M (EF3), 0.2 M (EF4), and 0.5 M NaCl (EF5) (each for 9 minutes), which resulted in separation of 5 elution peaks in addition to a peak containing non-bound material that was termed FT (flow through) (Figure 1). The content of separate chromatographic fractions was analyzed by assessing their toxicity, by detecting the presence of Atxs and Atns by ELISA, and by analyzing the purity and mass distribution of components by SDS-PAGE.

EF2 and EF3 were toxic in the higher test dose (20 μg/mouse), since all four mice died. The rest of EFs as well as FT were not toxic in the doses tested and no deaths were recorded.

ELISA analysis (Figure 2) showed, at first, that anti-AtxA and anti-$AtnI_2$ had no cross reactivity, so they could be used as tools for identification of respective venom components in separate peaks. It was observed that the whole content of Atxs was retained on the disk, since anti-AtxA didn't recognize anything in the peak FT. Anti-AtxA antibodies gave strong positive

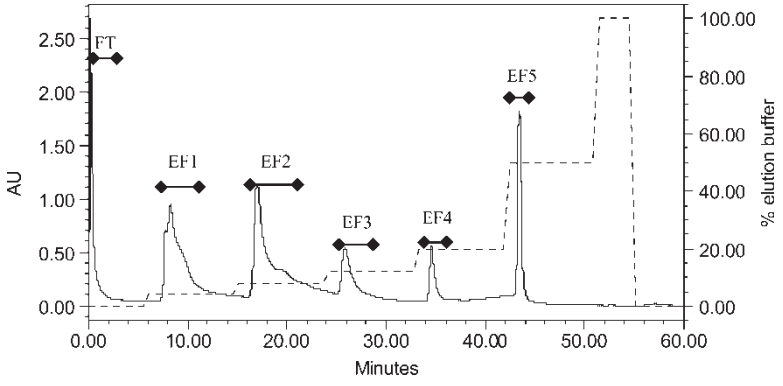

Figure 1. Chromatography of KR95 venom in 50 mM Tris/HCl buffer, pH 9.0. Elution was performed with stepwise NaCl gradient. Full line denotes absorbance at 214 nm, dotted line denotes NaCl gradient steps: 0.04 M (9th min), 0.08 M (18th min), 0.12 M (27th min), 0.2 M (36th min), 0.5 M (45th min), 1 M (53rd min). Elution buffer was 1 M NaCl in Tris/HCl buffer.

reaction with material in EF2 and EF3 and to a lesser extent in EF1 and EF4. Anti-$AtnI_2$ antibodies gave positive reaction only with material in FT.

SDS-PAGE (Figure 3) revealed that concentrated EF1-3 contained two matching, clearly visible bands of different molecular masses: $\approx$15 kDa and $\approx$100 kDa. EF1 had additional bands of $\approx$7 kDa and $\approx$35 kDa, while EF2 and EF3 contained predominantly 15 and 100 kDa bands. EF4 contained material of cca 25 kDa, and EF5 contained several bands of smaller molecular weight. At this moment, based on toxicity data, ELISA, and SDS-PAGE data, we knew that the band at 15 kDa in EF 1, 2, and 3 corresponded to Atxs, but we did not know the distribution of a particular Atxs among the three peaks.

The established chromatographic conditions were used for all subsequent analyses, only the duration of each elution step was shortened from the original 9 minutes (in Figure 1) to 6 minutes for the chromatography of pure Atxs as well as for the chromatography of smaller quantities of venom (4 mg/run) (all figures except Figure 1).

HPLC of Pure Basic $sPLA_2$s using CIM CM Disk under Established Experimental Conditions

Pure AtxA, B, and C, as well as pure AtnL, were dissolved in Tris/HCl buffer and 100 μg/run was applied to the CIM CM disk (AtxA and AtxC 100 μg/run, Atx B and AtnL 50 μg/run). Chromatography of each Atx and AtnL separately (Figure 4) revealed that AtxC and AtxB were eluted in the first gradient step (EF1; 0.04 M), AtxA was eluted in the second gradient step (EF2; 0.08 M), while AtnL was eluted in the fifth gradient step (EF5;

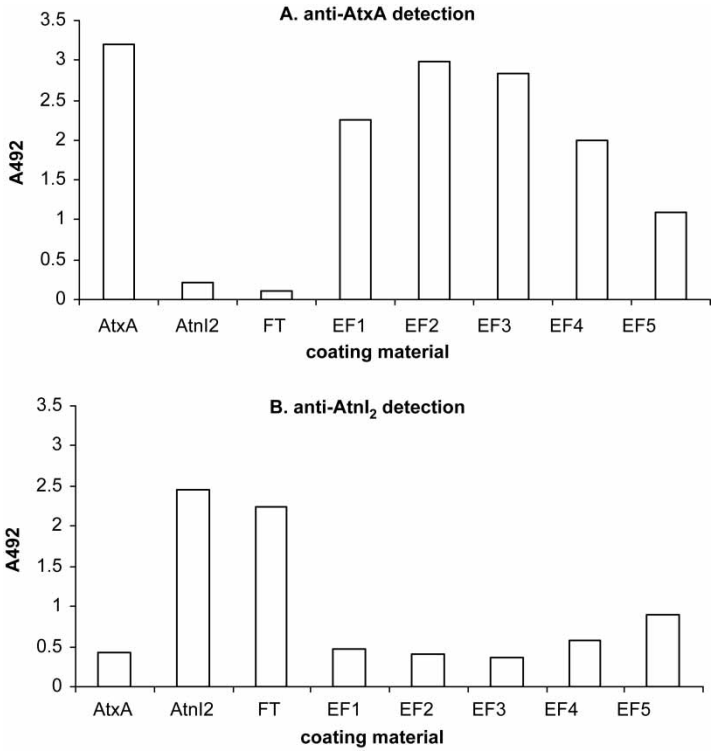

Figure 2. Analysis of distribution of $AtnI_2$- and AtxA-cross reactive material in chromatographic fractions by ELISA. Wells of ELISA plates were coated with material as denoted on x-axis, specific rabbit anti-AtxA (A) or anti-$AtnI_2$ (B) were added in the second step and positive reaction visualized by HRP-anti-rabbit IgG and addition of HRP substrate in the final step. Absorbance at 492 nm was measured.

0.5 M). Co-chromatography of the venom SL05 and pure AtxA (Figure 5) resulted in an increased signal at the position of EF2, testifying that AtxA is eluting only in this fraction.

HPLC of Two Batches of Long-Nosed Viper Venom and of *V. berus* Venom under Established Experimental Conditions

Each venom was dissolved in Tris/HCl buffer, pH 9.0, and 4 mg/run was applied to the CIM CM disk. Two batches of the long-nosed viper venom, SL05 and KR95, were run six times to determine the reproducibility of the established analytical procedure. Peak areas of each fraction were compared between all runs with the same venom sample, and the reproducibility expressed in terms of coefficients of variation (CV) calculated as the ratio of the mean value/standard deviation obtained for each elution fraction

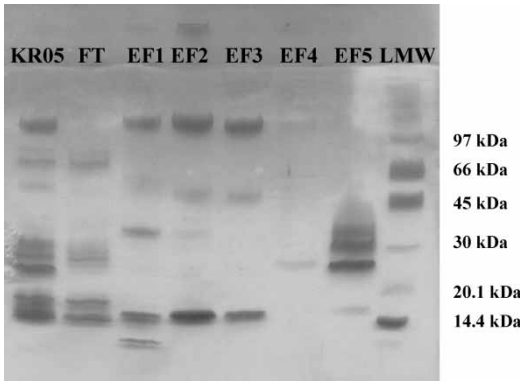

Figure 3. SDS-PAGE on 8–25% gradient gel of venom (KR05) and separated fractions after chromatography: flow through fraction (FT) and five concentrated elution fractions (EF1-5). LMW is low molecular weight markers kit.

(Table 1). The reproducibility of the method, with respect to the calculated CVs, was satisfactory. Chromatograms (Figures 6A and B) showed that the two batches of the *V. a. ammodytes* venom were quite similar in peaks EF4 and EF5 (regarding the shape and height of the peaks), but different in the first three peaks. The most pronounced difference was in the height of the peak 2, which was very high for the venom SL05 and significantly lower in KR95. To express these observations quantitatively, peak areas of each elution fraction obtained after chromatography of KR95 and SL05, respectively, were compared (Table 2). Results showed that EF2 peak area of SL05 was 2.5 times larger than EF2 peak area of KR95, EF1 peak area of SL05 was 1.65 time larger that of KR95. The differences in the EF3, EF4, and EF5 peak areas of KR95 and SL05 were small.

The chromatogram of *V. berus* venom (Figure 6C) differed significantly from the other two, lacking almost completely the peak EF3 and having a very small peak of EF2.

Comparison of Biological Properties of Venom Batches KR95 and SL05 and Correlation to the Respective Chromatograms

Venom SL05 had a two-fold higher toxicity than KR95, in terms of *i.v.* LD_{50} determined. SL05 had 180 LD_{50}/mg, and KR95 had 90 LD_{50}/mg. The ability of each venom to induce antibodies to AtxA was determined by ELISA analysis of immunized rabbit sera (Table 3). Rabbits immunized with SL05 developed significantly higher ($p = 0.05$) quantities of anti-AtxA IgG in comparison to rabbits immunized with the KR95 venom. Therefore, the two venom batches differed significantly in two biological properties measured (toxicity and potential for anti-AtxA induction in experimental animals) that both

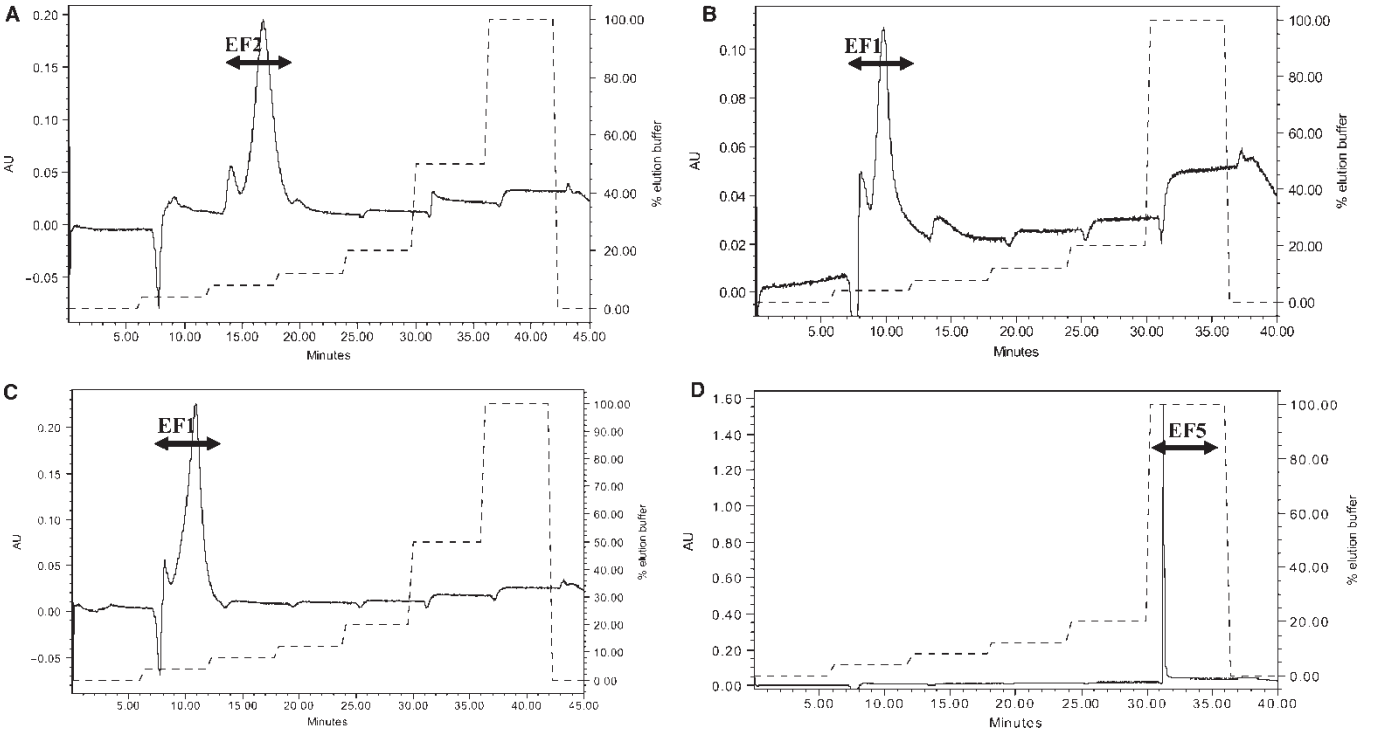

Figure 4. Chromatograms of 100 μg AtxA (A), 50 μg AtxB (B), 100 μg AtxC (C), and 50 μg AtnL (D) under established chromatographic conditions. Full lines denote absorbance at 214 nm. Dotted lines denote NaCl gradient steps: 0.04 M (6th min), 0.08 M (12th min), 0.12 M (18th min), 0.2 M (24th min), 0.5 M (30th min), 1 M (36th min).

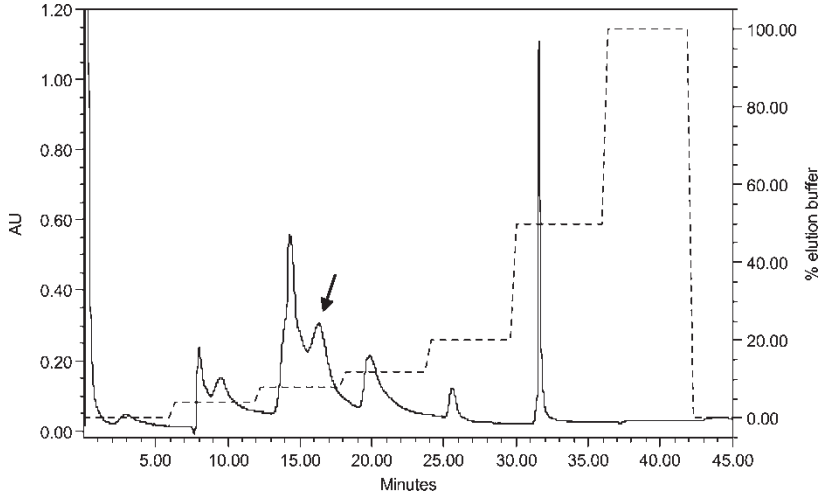

Figure 5. The chromatogram of the mixture of venom (1 mg) and AtxA (0.1 mg) in 50 mM Tris/HCl buffer pH 9.0. Full line denotes absorbance at 214 nm, dotted line denotes NaCl gradient steps: 0.04 M (6th min), 0.08 M (12th min), 0.12 M (18th min), 0.2 M (24th min), 0.5 M (30th min), 1 M (36th min). Additional elution maximum within EF 2 is denoted by arrow.

depend on the AtxA content of the venom. In agreement with this, the chromatograms of the two batches (Figures 6A and B) differed considerably in the shape and the height of the peak EF2, containing the majority of AtxA from the venom.

Table 1. Precision of the novel method estimated by the coefficients of variation of each elution fraction peak area obtained for KR95 and SL05 venom batch in six separate runs (4 mg/run)

Elution fracion	Peak area					
	KR95			SL05		
	Mean[a]/10^6	Sd[b]/10^6	CV[c]	Mean/10^6	Sd/10^6	CV
EF1	60.9	3.92	6.4	100.4	7.26	7.2
EF2	58.5	3.80	6.5	147.3	8.41	5.7
EF3	57.9	2.71	4.7	72.4	2.60	3.6
EF4	10.1	1.13	11.1	13.4	1.04	7.7
EF5	23.5	1.08	4.6	21.4	0.70	3.3

[a]Aritmetic mean of results from six runs.
[b]Standard deviation.
[c]Coefficient of variation calculated as Sd/Mean.

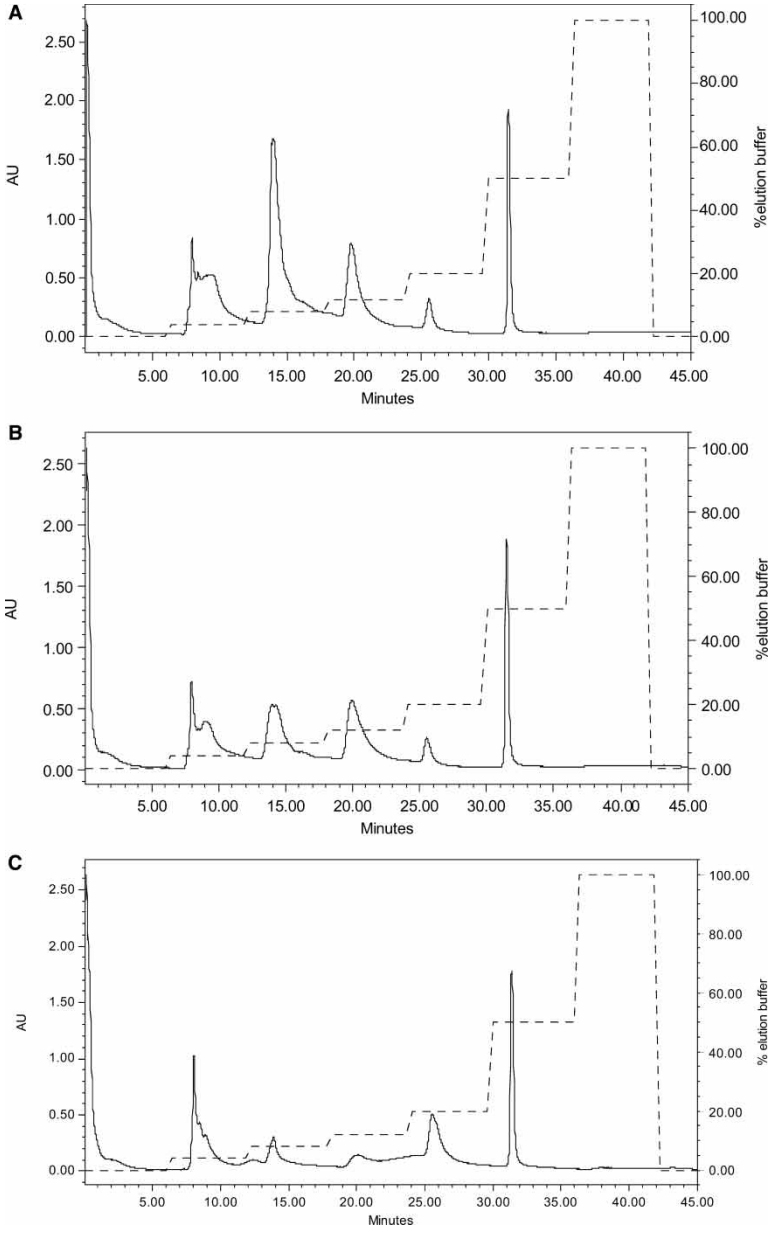

Figure 6. Chromatograms of two batches of *V.a. ammodytes* venom (A-SL05; B-KR95) and of *V. berus* venom (C). Venom, 4 mg of each, in 50 mM Tris/HCl buffer pH 9.0 was applied to the disk, and fractions eluted with NaCl gradient steps: 0.04 M (6th min), 0.08 M (12th min), 0.12 M (18th min), 0.2 M (24th min), 0.5 M (30th min), 1 M (36th min). Full line denotes absorbance at 214 nm, while dotted line presents NaCl gradient steps.

Table 2. Comparison of mean peak areas of respective elution fractions obtained after chromatography of SL05 and KR95 venom batches (4 mg/run)

Elution fractions	Peak area/10^6		
	KR95	SL05	SL05/KR95
EF1	60.9 (±3.92)	100.4 (±7.26)	1.65
EF2	58.5 (±3.80)	147.3 (±8.41)	2.52
EF3	57.9 (±2.71)	72.4 (±2.60)	1.25
EF4	10.1 (±1.13)	13.4 (±1.04)	1.32
EF5	23.5 (±1.08)	21.4 (±0.70)	0.91

DISCUSSION

Snake venoms are very complex mixtures of numerous components with different biochemical properties and biological activities. The analyses of such materials and even the partial separation of components, or the group of components, require strategies focused on their particular properties.

Table 3. The quantity of anti-AtxA IgG in individual sera of rabbits immunized with the same quantity of SL05 and KR95 venom, determined by ELISA, and expressed in arbitrary unit (AU)/ml. Median values are given in bold. Anti-Atx IgG values obtained in rabbits immunized with the two venom batches were significantly different at $p = 0.009$ (data analyzed by Mann-Whitney U-test)

Antigen for immunisation	Anti-AtxA IgG (AU/mL)
SL05	8267
	16716
	5708
	7477
	15378
	8267
KR95	1216
	3069
	4305
	3312
	986
	3069

The variability in biochemical properties and biological activity of *V. a. ammodytes* venoms collected from snakes originating from different geographical regions[17] and from snakes milked in different seasons of year has already been reported.[18] The different batches of venom prepared from snakes from different regions of Croatia, have empirically been shown as not equally good antigens for antivenom production (personal communication). Also, we have recently noticed in rabbits that the venom toxicity neutralizing efficacy of the venom specific rabbit antisera is highly correlated to the level of IgG specific to Atxs (Halassy et al. in preparation). We further observed and presented here that two venom batches, originating from different regions, differ in their ability to induce antibodies specific for AtxA in rabbits. All these data indicated that the analysis of venoms with respect to their basic proteins and Atx content might be used for screening of venoms, prior to their use as potential antigens in immunization of experimental animals.

The aim of this work was, therefore, to establish a fast and simple method for separation of basic proteins (comprising Atxs) from *V. a. ammodytes* venom and to investigate whether the method could be used for analysis of venom batches regarding their antigenic potential. Conventional procedures for separation of basic $sPLA_2$s from this venom have been described, but we considered them too sophisticated and time consuming for routine comparison of different venom batches.[8,13] We have shown that chromatography of whole venom on the CIM CM disk using HPLC resulted in satisfactory extraction of a group of basic components, and in their subsequent partial separation according to different pI values. Separated peaks were analyzed by SDS-PAGE and further characterized by testing the toxicity and reactions with specific antisera. Toxic components were found in peaks eluted with lower concentrations of NaCl. Proteins eluted in these peaks reacted with anti-AtxA in relevant ELISA tests. Non-toxic $sPLA_2$s with neutral pI values, including $AtnI_2$, were not retained on the disk, as anti-$AtnI_2$ did not react with material in bound fractions. Chromatography and analysis of AtxC (pI 9.4), AtxB (pI 10.0), AtxA (pI 10.2), and AtnL (pI $>$ 10.5) separately revealed that under experimental conditions AtxC and AtxB were eluted in the first NaCl gradient step (EF1), AtxA as the most basic Atx in the second NaCl gradient step (EF2), while AtnL was eluted in the final NaCl gradient step (EF5).

On the basis of these results two batches of the long-nosed viper venom were chromatographically analyzed and compared. These two batches differed in toxicity and anti-AtxA IgG inducing capacity, the properties related to their AtxA content. The most pronounced difference in their CIM CM disk chromatograms was in the height and shape and, consequently, peak area of the peak EF2, which also contained AtxA. According to our preliminary results, the content of AtxA in the venom is particularly important for induction of venom protective antibodies.

It has to be noted that separation and isolation of snake venom $sPLA_2$s have been constantly reported since these bioactive peptides have been very

interesting for many reasons, for example, the neurotoxic $sPLA_2$s have been extensively investigated as promising tools for studying various aspects of nerve function and dysfunction.[19,20] Also, because of their small size and versatile pharmacological activities they have been very good models for studies on protein structure functions relationship. Among the recently reported fast chromatographic methods for purification of $sPLA_2$s is the isolation of two new $sPLA_2$s from *Vipera nikoloskii* venom that includes the combination of cation exchange chromatography on HEMA BIO 1000 CM column and RP-HPLC,[21] and the one step RP-HPLC chromatographic procedure for the isolation of two $sPLA_2$ isoforms, Cdr-12 and Cdr-13, from *Crotalus durissus ruruima* snake venom.[22] The use of monolithic chromatographic support is an additional, very effective approach for analysis of snake venoms and purification of their components.

CONCLUSION

In this study, we have shown that chromatography on the CIM CM disk is a convenient method for separation of basic components from the snake venom. By adjusting the concentration of NaCl in discontinuous gradient, this group of compounds could be further separated. In this way, the content of Atx in different *V. a. ammodytes* venom batches can be rapidly assessed and compared. On the basis of our initial study, we recognized that the method may serve as an efficient tool to predict the antigenicity of a particular *V. a. ammodytes* venom batch. The results presented here are the first data on chromatographic analysis of snake venoms using monolithic chromatographic supports. Our findings open the possibilities for further improvements and modifications of the method and combination of different CIM disks in analyses of snake venoms.

ACKNOWLEDGMENTS

We sincerely thank Dr. Petra Prijatelj for her technical assistance. This work was supported by the grant BI-HR/06-07-008 from the Slovenian Ministry of Higher Education, Science, and Technology and Croatian Ministry of Science, Education, and Sports and by the grants 021-0212432-2033, 021-0212432-2431, and TP-05/0021-01 from the Croatian Ministry of Science, Education, and Sports.

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Received March 15, 2007
Accepted May 18, 2007
Manuscript 6172E