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Adenosine in the venoms from viperinae snakes of the genus *Bitis*: Identification and quantitation using LC/MS and CE/MS

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Abstract

Snake venoms are rich sources of toxic proteins and small molecules. This study was directed at molecules of molecular mass below 1 kDa. Thirty different venoms, of either neurotoxic or haemorrhagic type, were fractionated using size-exclusion chromatography. Only venoms of the Puff adder (*Bitis arietans*), Gaboon viper (*Bitis gabonica*), and Rhinoceros viper (*Bitis nasicornis*) exhibited large absorbance peaks at λ_{280nm} in the total volume range of the chromatographic column indicating the presence of abundant low molecular mass material. Analysis of fractions containing this material using both HPLC and capillary electrophoresis interfaced with electrospray ion-trap mass spectrometry unequivocally established that the bioactive nucleoside, adenosine, was the major component. The concentrations of adenosine found (Puff adder—97.7×10^{-6} mol L^{-1}; Gaboon viper—28.0×10^{-6} mol L^{-1}; and Rhinoceros viper—56.8×10^{-6} mol L^{-1}) were above those required to activate all known sub-types of adenosine receptors. Adenosine may thus act at the site of envenomation causing local vasodilatation and may play a role in the subsequent systemic hypotension observed.

Keywords: Adenosine; Snake venom; Mass spectrometry

Venoms and toxins of natural origin have long fascinated man as a result of their profound pharmacological effects. Venoms are complex mixtures of many different components, whereas a toxin is a single pure compound. Toxins often have novel, highly specific activities and are of interest as pharmacological tools due to their exquisite selectivity. Studies on toxins have often paved the way for the design of therapeutically useful molecules [1].

The venom-producing apparatus of snakes is composed of modified exocrine glands that produce a complex cocktail of toxic proteins and polypeptides, enzymes, enzyme inhibitors, and peptides with diversified physiological activities [1]. Proteins and peptides comprise between 90% and 95% of the dry weight of the venom and these include the metalloproteinases, phospholipases, acetylcholinesterase, and phosphodiesterase enzymes [2–4]. Other components found in venom are metallic cations, carbohydrates, nucleosides, biogenic amines, and low levels of free amino acids and lipids [5]. Snake venom research has undergone a renaissance with efforts directed towards the discovery and molecular characterization of novel molecules that may have commercial applications, particularly as clinical therapeutics. The best-known example of successful drug development from a component of snake venom

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is that of the inhibitors of angiotensin-converting enzyme (ACE). The lead compounds for these drugs were discovered from the bradykinin-potentiating peptides of *Bothrops jararaca*, the Brazilian arrowhead viper, and they have molecular masses between 500 and 1300 Da. Bradykinin is an endogenous nonapeptide that results in increased vascular permeability, dilation of blood vessels, and contraction of non-vascular smooth muscle [6]. Bradykinin is rapidly degraded, primarily in the pulmonary system, by the action of angiotensin-converting enzyme (ACE), carboxypeptidase N and endopeptidase [7]. Studies on snake venoms that showed blocking of plasmakinin degrading activity led Ferreira et al. [8] to isolate bradykinin-potentiating peptides from *B. jararaca* venom. These peptides were later shown to act by blockade of angiotensin-1 converting enzyme (ACE), that generated angiotensin-2, a vasoconstrictor, and degraded bradykinin, a vasodilator, thus producing an anti-hypertensive effect. Discovery and the functional study of bradykinin-potentiating peptides (BPPs, known also as angiotensin-converting enzyme inhibitors) in snake venom have been well documented and the utilization of BPP as a lead compound has contributed to the development of clinically important ACE inhibitor drugs [8–11].

To date, most studies directed towards the purification and characterization of snake venom components have concentrated on large molecular weight enzymes and toxic proteins [12]. This is most surprising considering the clinical and commercial importance of the ACE inhibitors that are derived from low molecular weight venom components. Thus, in this study, we concentrated chemical analysis on low molecular weight (less than 1 kDa) molecules in a broad spectrum of these venoms. Thirty snake venoms were subjected to separation by size-exclusion chromatography. However, only three venoms from the essentially African viperinae snakes of the genus *Bitis* exhibited large absorbance peaks in the low molecular weight region of the chromatograms. The material constituting these peaks was analysed using HPLC-MS/MS and capillary electrophoresis with UV diode-array detection. These analytical techniques unequivocally established the identity of the component as adenosine and the quantities present were within the concentration ranges that are known to be capable of effecting adenosine receptor activation.

Materials and methods

Reagents

All solvents were of HPLC grade, Milli-Q water (Millipore) was used throughout, and all other chemicals used were of analytical reagent quality. Methanol, acetonitrile, and acetic acid were obtained from BDH (Poole, Dorset, UK), while citric acid was obtained from Sigma (Poole, Dorset, UK). Adenosine was obtained from Sigma (Poole, Dorset, UK) and a standard solution was prepared by dissolving an appropriate mass in 25 ml water to provide a concentration of 1.0×10^{-3} mol L^{-1}. Nitrogen gas for the LCQ was delivered from a Whatman nitrogen generator (Whatman, Haverhill, MA, USA) while helium-damping gas, present in the ion trap, was obtained from BOC Gases (Guildford, Surrey, UK).

Acquisition of venoms

All venoms were collected from authentic identified snake species housed in the Venom Unit of the Liverpool School of Tropical Medicine (Liverpool, UK), and were supplied in lyophilized form (5–15 mg dry weight).

Instrumentation

Size-exclusion chromatography. Lyophilized venom samples were applied to a Sephadex G-50 column (1 cm × 90 cm) (Pharmacia, Sweden) and eluted at a flow rate of 10 ml h^{-1}. Fractions of 2 ml were collected at 10 min intervals and the column effluent was continuously monitored at a wavelength of 280 nm using a flowthrough spectrophotometer.

MS^n characterization. MS^n characterization was performed using an LCQ quadrupole ion-trap mass spectrometer (Thermo Electron Corporation, San Jose, California, USA) utilizing electrospray ionization (ESI). Samples were introduced either by means of a syringe pump located on the instrument or online following HPLC separation.

High performance liquid chromatography–mass spectrometry (LC/MS). The HPLC system used was supplied by Thermo Electron Corporation (San Jose, California, USA) and comprised a SCM 1000 vacuum membrane degasser, P4000 pump, AS 3000 autosampler, and online UV 1000 UV detector. The column used for chromatographic separations was a Phenomenex (Macclesfield, Cheshire, UK) Luna C_{18} column; 250 mm × 4.6 mm. A Security Guard cartridge (Phenomenex, Macclesfield, Cheshire, UK) was positioned just before the analytical column. For mass spectrometric studies, the HPLC system was interfaced with the LCQ instrument (Thermo Electron Corporation, San Jose, California, USA).

Capillary electrophoresis. Conventional capillary electrophoresis (CE) investigations with UV detection were carried out using a Spectrophoresis CE 1000 instrument manufactured by Thermo Electron Corporation (San Jose, California, USA). For UV detection studies, separations were achieved using an untreated fused silica capillary 700 mm × 75 μm (Composite Metal Services, Hallow, Worcestershire, England). A window was burned in the polyimide coating at 58 cm, with detection taking place by means of a diode-array detector (Thermo Electron Corporation, San Jose, California, USA). Spectacle software (Thermo Electron Corporation, San Jose, California, USA) was used to store and analyse the UV profiles generated.

Procedures

Sephadex G-50 fractionation of snake venoms. Lyophilized snake venoms were reconstituted in 1 ml of 2 mol L^{-1} acetic acid and applied to the top of the column. The same acid solution was used as eluant at a flow rate of 10 ml h^{-1}. Fractions (2 ml) were collected at 12 min intervals using an automated fraction collector. Insulin (5800 Da), cytochrome *c* (12,380 Da), and trypsin (23,800 Da) were employed as molecular weight markers for calibration of the Sephadex column: V_o (void volume) was measured by Blue Dextran (200 kDa) and V_t (total volume) by using potassium dichromate (320 Da). A linear relationship of $\log M_w$ versus elution volume was observed.

MS^n characterization of adenosine. A 1.0×10^{-5} mol L^{-1} standard of adenosine was infused into the LCQ via a syringe pump at a rate of 10 μl min^{-1}. Nitrogen sheath and auxiliary gas flows were set to 50 and 5, respectively (arbitrary values set by the software), while the capillary

temperature was maintained at 250 °C and the spray voltage at 5.0 kV. Using the LCQ software, the optimized collision energy value at each stage of fragmentation was obtained. The procedure was repeated for pooled snake venom fractions.

High performance liquid chromatography–mass spectrometry (LC/MS). A mobile phase compatible with the electrospray process was used and consisted of 69.5:30.0:0.5 (v/v/v) water:methanol:acetic acid. Isocratic elution was employed at a flow rate of 0.5 ml min^{-1}. Injections of 50 μl aliquots of standards and samples were made via the autosampler. The LCQ parameters were identical to those described for MS^n characterization above, except that the sheath and auxiliary gas flows were raised to 65 and 30, respectively, to account for the higher solvent flow rate. Calibration, RSD, and LOD data were obtained and quantitation was carried out by comparing the peak area of adenosine present in the venom samples with the peak area of standard concentrations of adenosine in the linear range of the calibration plot.

Capillary electrophoresis. CE was also used to identify adenosine in snake venom fractions using diode-array detection. The capillary was equilibrated with 0.1 mol L^{-1} NaOH at 60 °C for 5 min after which the capillary was flushed with Milli-Q water, also at 60 °C for 5 min followed by a further 5 min 0.1 mol L^{-1} NaOH wash at 30 °C. The capillary was then rinsed with run buffer (0.02 mol L^{-1} citric acid with 15% methanol, the pH of the solution being 2.50) at 30 °C for 5 min. Conditioning then took place by applying the run potential to the buffer-filled capillary until a constant run current was achieved. A separation potential of 20 kV was used, the temperature maintained at 30 °C and the detector set to high-speed scanning mode between 210 and 310 nm with monitoring taking place at 260 nm, the wavelength of maximum absorbance for the compound. Standards were prepared in Milli-Q water by serially diluting a 1.0×10^{-3} mol L^{-1} aqueous stock solution of adenosine. Initially a 1.0×10^{-4} mol L^{-1} standard of adenosine was analysed and its resulting UV profile library was stored for comparison with venom samples as a qualitative determination for the presence of the compound. Calibration, RSD, and LOD data were obtained, however for quantitation purposes HPLC-MS/MS was preferred.

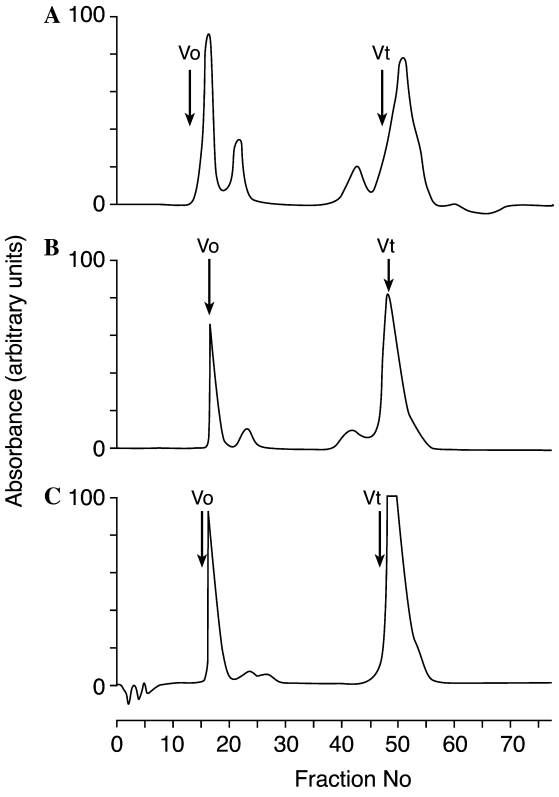

Fig. 1. Sephadex G50 gel filtration profiles of (A) *B. nasicornis* venom, (B) *B. arietans* venom, and (C) *B. gabonica* venom. Column effluent was monitored spectrophotometrically at λ_{280nm}, and both the void (V_o) and total volumes (V_t) of the chromatographic column are indicated.

Results and discussion

Venom fractionation via size-exclusion chromatography

Thirty different snake venoms from a selection of species from the families *Elapiidae* and *Viperiidae* were fractionated using size-exclusion chromatography (complete data set not shown). In only three venoms, from the essentially African vipers, *Bitis gabonica*, *Bitis nasicornis*, and *Bitis arietans* (Fig. 1), were any major absorbance peaks observed in the UV trace corresponding to low molecular weight molecules. All three size-exclusion-profiled venoms displayed major absorbance peaks in the high and low molecular weight regions. In *B. nasicornis* (Fig. 1A) and *B. arietans* (Fig. 1B), two peaks were observed in the low molecular weight region, one being significantly larger than the other.

MS^n fragmentation

Direct infusion of the low molecular weight fractions from the three venom samples into the electrospray mass spectrometer produced a precursor ion peak at *m*/*z* 268. MS/MS of the 268 precursor resulted in a product ion of *m*/*z* 136 (Fig. 2A). No further fragmentation products were observed, at the MS^3 stage. Adenosine having a molecular mass of 267 was selected for investigation. With direct infusion of adenosine standard onto the mass spectrometer, a signal at *m*/*z* 268 was observed in MS corresponding to the $[M + H]^+$ species. MS/MS of the adenosine 268 ion resulted in a product ion of *m*/*z* 136, resulting from loss of the entire sugar moiety and leaving the protonated adenine group. No further fragmentation products were observed, possibly due to the protonated entity being lost at the MS^3 stage thus giving an uncharged product that is not detected in the ion trap. The MS/MS profile with suggested structures for each stage of MS is shown in Fig. 2B. We propose that protonation occurs at the tertiary amine on the adenine group and that this protonated site is retained throughout the rest of the fragmentation steps, eventually being lost at MS^3.

Capillary electrophoresis

Adenosine has been reported to have pK_a values of 3.6 and 12.4, attributable to the adenine and ribose groups, respectively [13]. At a run buffer pH of 2.5, it would thus be expected that adenosine would be a singly

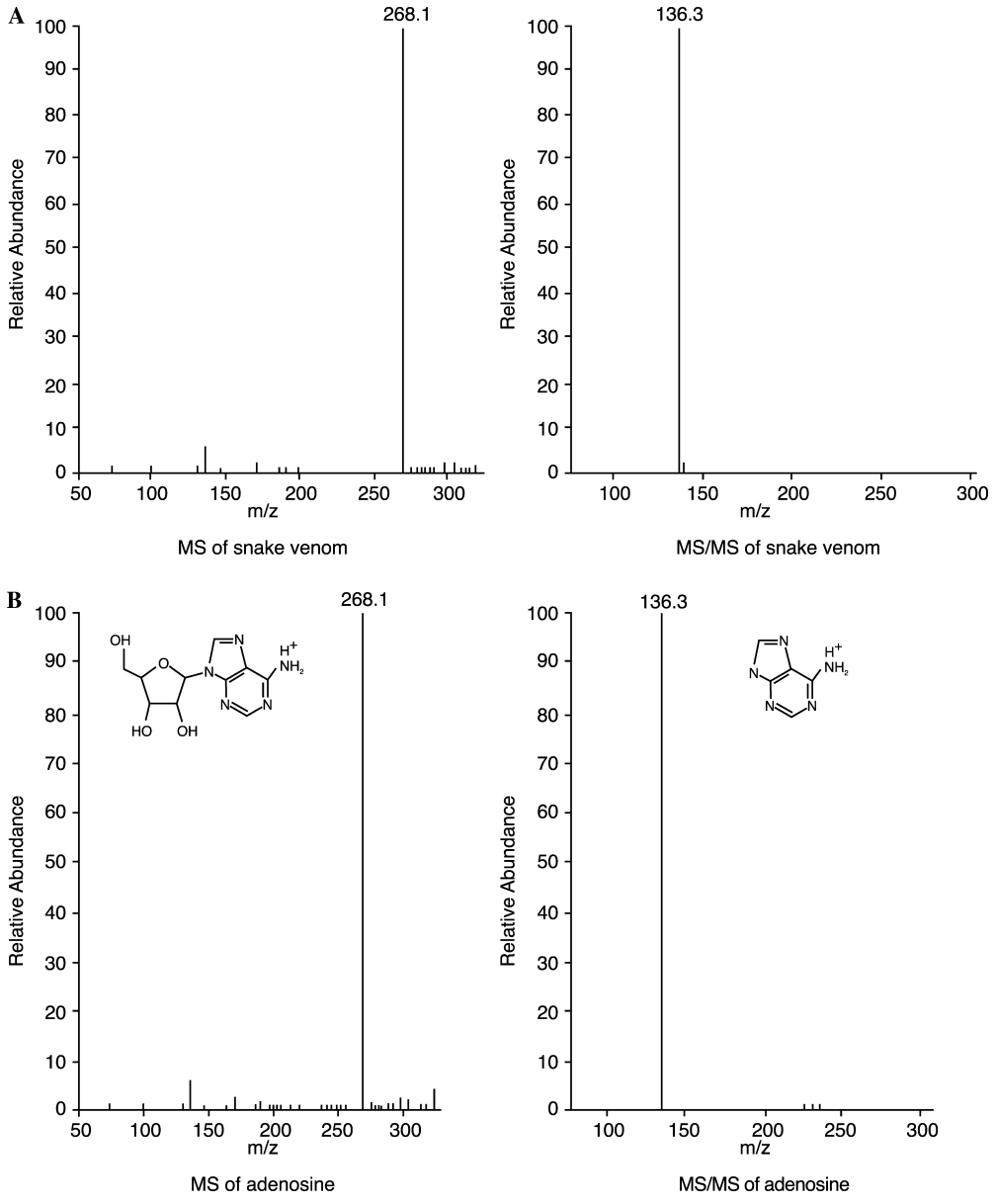

Fig. 2. (A) Fragmentation profile of snake venom samples showing the 268 *m*/*z* precursor ion peak observed upon initial infusion of snake venom samples into the mass spectrometer along with the 136 *m*/*z* product ion peak observed in MS/MS analysis. (B) Fragmentation profile of adenosine standard showing the 268 *m*/*z* precursor ion peak observed upon initial infusion of adenosine into the mass spectrometer along with the 136 *m*/*z* product ion peak observed in MS/MS analysis.

protonated species and migrate under its own electrophoretic mobility in the direction of the cathode. Using the scanning diode-array detector, the adenosine standard was found to have a maximum absorbance at a wavelength of 260 nm and its capillary electrophoresis profile was stored for reference using Spectacle software. Neat samples of the venom fractions were then injected onto the capillary and analysis took place. While capillary electrophoresis suffers from relatively poor LODs compared to HPLC-MS, adenosine was readily detectable in all the venom samples, indicating that a high concentration of the compound was present. Adenosine had a typical migration time of 12 min with a daily fluctuation of ±2 min. Positive identification was not based solely on migration time, which is known to shift in CE, but also by comparison with the library-stored UV profile. As the fractions analysed had already undergone purification by size-exclusion chromatography, the electropherogram in each case contained only one signal identified as adenosine. Fig. 3 shows the CE electropherogram peak for standard adenosine and the adenosine found in *B. arietans*. The two venoms from *B. gabonica* and *B. nasicornis* displayed similar signals (data not shown). Following each analysis of venom, the trace

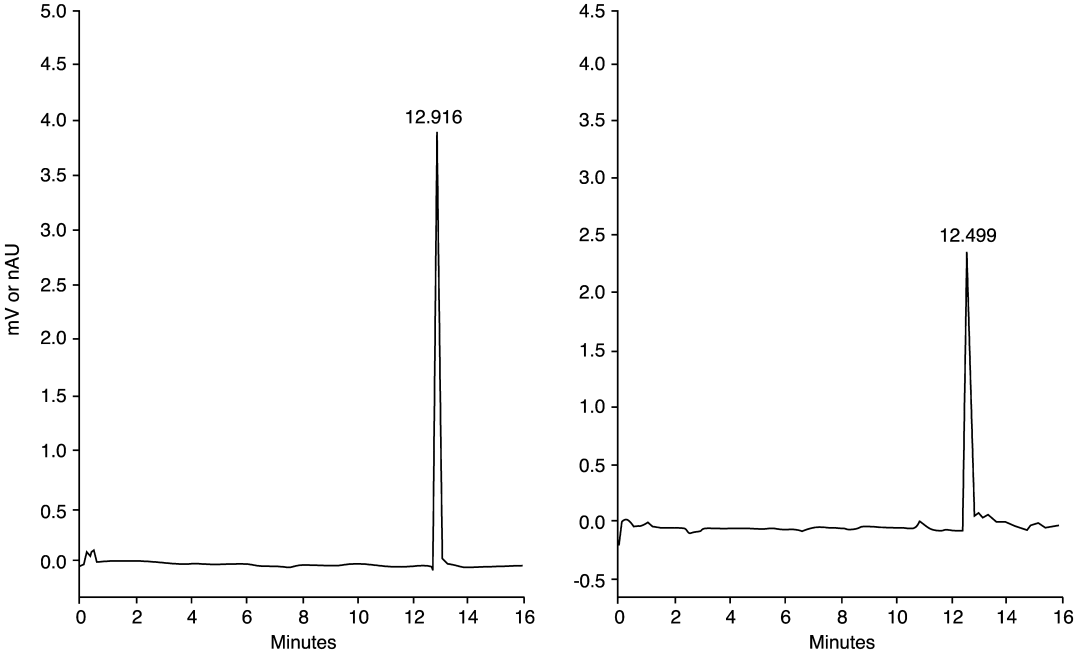

Fig. 3. The capillary electrophoresis electropherogram peak for adenosine standard (left panel) and the adenosine present in *B. arietans* venom (right panel).

was compared with the stored library profile and a match coefficient produced for each. They were 981.4, 989.9, and 986.0 for *B. arietans*, *B. nasicornis*, and *B. gabonica*, respectively, indicating a close match for adenosine in each case, with a value of 1000 corresponding to an identical match.

High performance liquid chromatography–mass spectrometry (LC/MS)

When LC/MS was employed for the analysis of adenosine standard and the proposed adenosine occurring in venom samples, both exhibited retention times of 5 min. Additionally, in MS/MS fragmentation mode, both generated the characteristic *m/z* 136 product ion, shown for *B. arietans* in Fig. 4. For quantitative purposes, the venom samples were diluted fourfold in methanol for *B. gabonica*, 40-fold for *B. nasicornis*, and one hundred-fold for *B. arietans*, to ensure that the observed signal lay within the linear calibration range for adenosine. Table 1 compares analytical data generated by HPLC-MS/MS and capillary electrophoresis UV diode-array. Both HPLC-MS/MS and CE–UV gave acceptable relative standard deviation (RSD) values for retention and

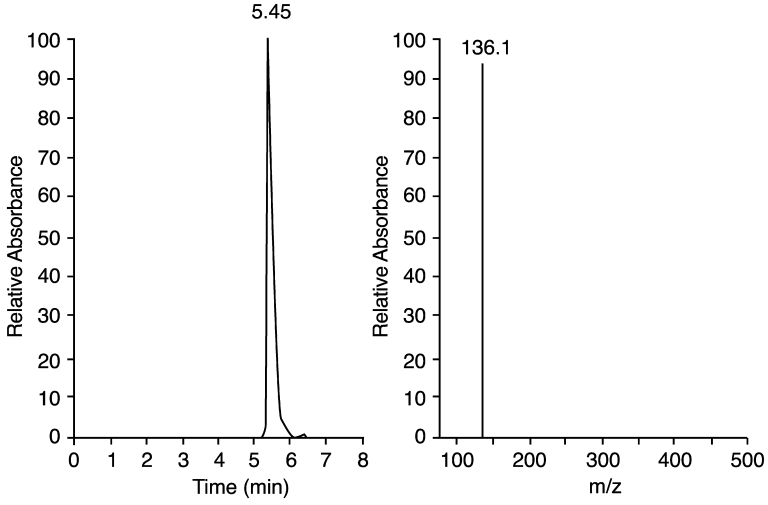

Fig. 4. HPLC MS/MS elution profile of adenosine in *B. arietans* venom. The left-hand trace corresponds to the UV chromatogram at 280 nm absorbance for adenosine, while the right-hand trace shows the 136 *m/z* product ion in MS/MS.

Table 1
Analytical data for the determination of adenosine standards ($n = 6$) by CE and HPLC MS/MS

Technique	Concentration (mol L^{-1})	Retention time (min)	RSD (%)	LOD (mol L^{-1})
HPLC MS/MS	2.5×10^{-6}	5.27	0.68	2.5×10^{-8}
CE UV	1.0×10^{-4}	10.48	1.96	3.0×10^{-6}

migration times for adenosine of 0.68% and 1.96%, respectively. Relative standard deviation values for peak area were also within acceptable limits, and calibration data over 1 order of magnitude gave R^2 values of 0.9981 and 0.9963 for HPLC-MS/MS and CE, respectively. As expected, HPLC-MS/MS proved to be the more sensitive technique having a limit of detection around two orders of magnitude lower than that achieved by CE. HPLC-MS/MS was therefore used as the quantitative tool for these studies. For the determination of adenosine in snake venom, multiple injections were made for each sample as shown in Table 2. Three injections of methanol were made between each sample set to ensure that carry-over did not occur. A known mass of starting material allowed for the calculation of the concentration of adenosine in each of the venom samples. These values are also shown in Table 2 and may be translated as 0.152, 0.162, and 0.025 μg mg^{-1} adenosine in venom from *B. nasicornis*, *B. arietans*, and *B. gabonica*, respectively. The RSD values for peak area were 1.68%, 2.83%, and 2.75%, respectively.

Utilising the high specificity of HPLC-MS/MS and capillary electrophoresis technologies we have definitively proven that the large absorbance peak observed within the size-exclusion chromatography profiles, in the small molecular weight region of the three *Bitis* venoms, is due primarily to the nucleoside adenosine. Coupling HPLC with electrospray ion trap mass spectrometry enabled identification and quantitation of the amounts of adenosine present in these three snake venoms.

The prey of *Bitis* snakes are subjected to massive hypotension upon envenomation [14,15]. From this study, we attribute the hypotensive effect of the *Bitis* venom to be in part due to the activity of the high concentration of adenosine present. The vasodilator effects of adenosine and adenine nucleotides have been known for some time, being first recognized by Drury and Szent-Gyorgi in 1929 [16]. We suggest a putative role for adenosine and its secondary metabolite, inosine, in the hypotension observed in the prey of these snakes via their interaction with endogenous adenosine receptors. The action of adenosine is mediated through G-protein-coupled receptors classified into four subtypes, A_1, A_{2A}, A_{2B}, and A_3, on the basis of their affinity order profiles for agonists and antagonists [17,18]. The A_1 and A_{2A} receptors are activated by submicromolar concentrations of adenosine whereas the A_{2B} and A_3 receptors are activated only when adenosine levels rise into the micromolar range [19]. The concentrations of adenosine observed in this study are above the micromolar range and thus sufficient to activate all receptor sub-types. The role of adenosine in the inhibition of neurotransmitters at both the central and peripheral nerve terminals has been known for the past three decades. For example, activation of A_1 receptors by adenosine suppresses acetylcholine release from the motor neurons [20]. Adenosine also exacerbates venom-induced hypotension by activating A_2 receptors producing vasodilatation and increasing capillary permeability in the vasculature [19,21,22]. Jin et al. [23] demonstrated that adenosine and its secondary metabolite, inosine, trigger mast cell degranulation by acting on the A_3 receptor. Mast cells are present in most tissues, including the heart, brain, lungs, and kidneys, and are often found in close proximity to blood vessels, including capillaries and post-capillary venules. Upon stimulation these cells release a number of mediators that include leukotrienes, histamine, and serotonin, that all act directly on the vasculature to produce vasodilatation [19,24]. In rats, APNEA [*n*6-2-(4-aminophenyl)ethyladensoine], an adenosine agonist, also acts on the A_3 receptor on mast cells causing degranulation. This results in a dose-related fall in blood pressure of up to 30 mmHg, which lasts in excess of 30 min [25,26]. This body of evidence strongly supports our assertion that the levels of adenosine present in the venom of these three snakes are sufficient to play a major role in the severe hypotension observed upon envenomation.

Table 2
Adenosine concentrations in the venoms from three species of *Bitis* snakes ($n = 6$ in each case)

Snake	Measured concentration (mol L^{-1})	RSD	Mass of adenosine in venom (μg mg^{-1})
B. nasicornis	56.8×10^{-6}	1.68	0.152
B. arietans	97.7×10^{-6}	2.83	0.162
B. gabonica	28.0×10^{-6}	2.75	0.025

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