Venom:Antivenom Immune Complex Binding Assay Using Size-Exclusion High-Performance Liquid Chromatography (SE-HPLC)

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Abstract

The treatment of envenomation with effective snake antivenom immunoglobins has become a critical worldwide health issue. Current methods for testing the effectiveness of new antivenom mixtures in neutralizing venom toxicity/lethality use animal models (e.g. mice). Neutralization of venom toxicity/lethality requires the formation of venom-antivenom immune complexes (though the extent of complex formation *in vivo* is unknown). Size-exclusion high-performance liquid chromatography (SE-HPLC) is a reproducible quantitative method to characterize venom-antivenom immune complex formation *in vitro* within a relatively short time. Changes in SE-HPLC elution profiles due to dose-dependent formation of venom-antivenom immune complexes are presented for 1) *Crotalis atrox* (western diamondback rattlesnake) venom and the current antivenom used clinically in North America [F_{ab}AV (Ovine); CroFab™], and 2) *Bothrops jararaca* venom (Brazil) and Bothropic antivenom [F(ab')$_2$AV (Equine); Brazil]. Changes in profile region areas were fit to a hyperbolic dose-response function to estimate maximum binding and venom/antivenom concentrations at half-maximum binding.

Materials and Methods

Equipment:
The HPLC system consisted of a Waters (Milford, MA) Model 515 HPLC pump, a Waters 717plus Autosampler, a TSKgel G3000SW$_{XL}$ (5 µm, 7.8 mm ID x 30 cm) SEC column (TOSOH Bioscience), and a Waters 2996 Photodiode Array Detector. Detector response was recorded using Empower 3 (Waters) acquisition software at a 1 ml/minute flow rate at room temperature in elution buffer [100 mM sodium phosphate (pH 6.7) containing 0.10 M sodium sulphate].

Materials:
Antivenin: F_{ab}AV - Ovine F_{ab} fragments (CroTAB™, Protherics Inc., Brentwood TN,USA); $M_r \approx 50$ kDa; one binding site/molecule.

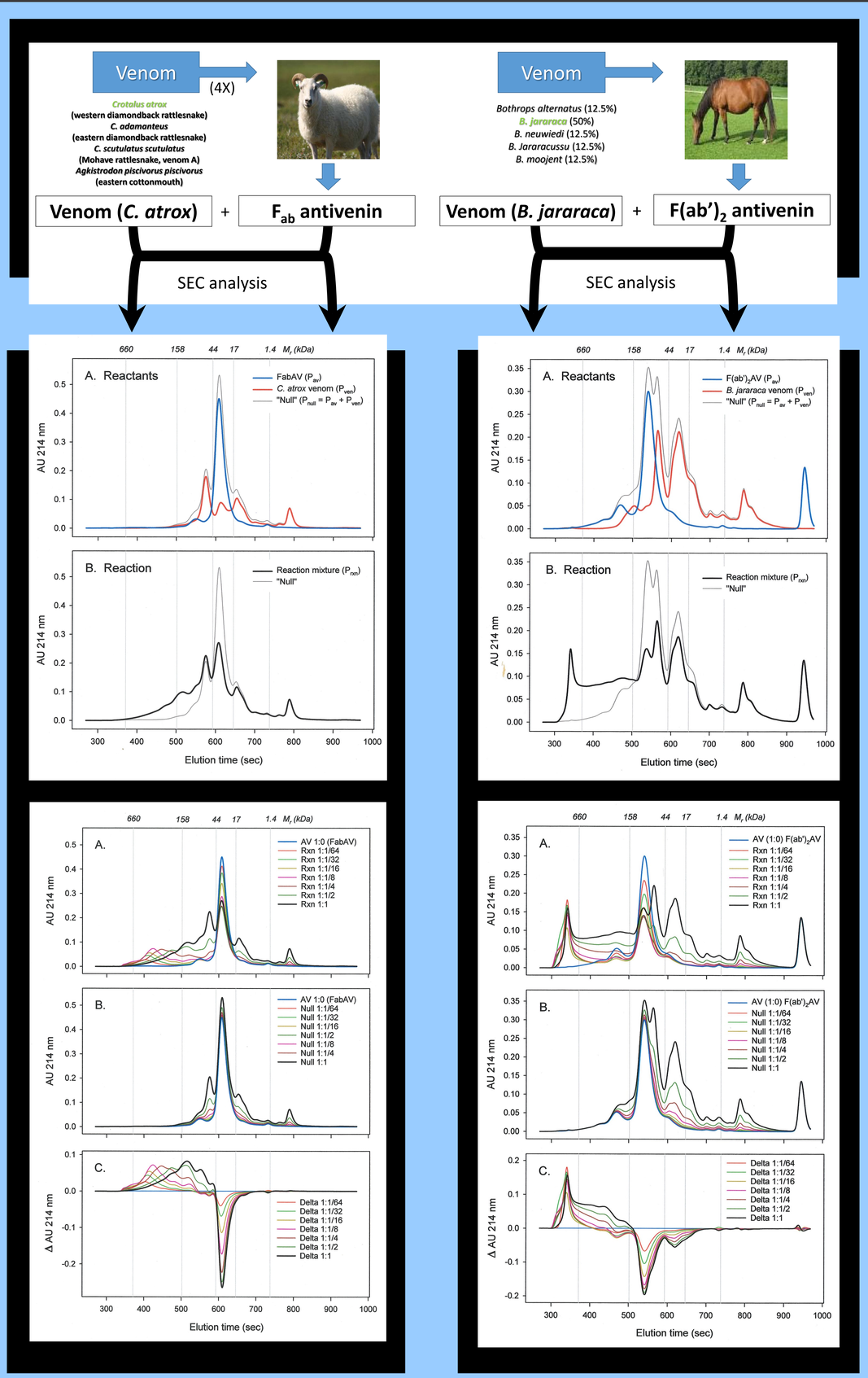
F(ab')$_2$AV - Equine F(ab')$_2$ fragments (Bothropic antivenom, Butantan Institute, San Paulo, SP, Brazil); $M_r \approx 100$ kDa; two binding sites/molecule.

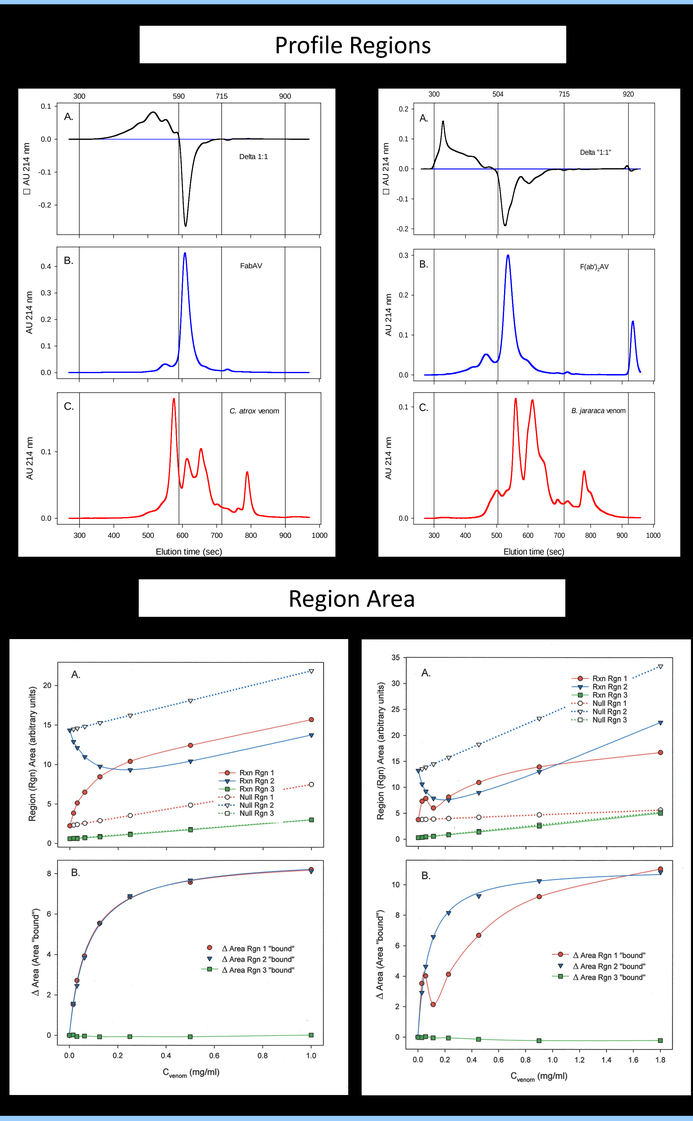
Venoms: *Crotalus atrox* (Western diamondback rattlesnake), Sigma (St. Louis, MO, USA)

Bothrops jararaca (Brazilian arrowhead viper), via Rita Collaço

Samples: Venom, antivenom, and mixtures of antivenom containing varying concentrations of venom were incubated at 37°C for 30 minutes prior to SEC analysis. Sample injection volume = 20 µl

Analysis: Changes in profile region areas were fit to hyperbolic dose-response functions using SigmaPlot to estimate maximum binding and venom/antivenom concentrations at half-maximum binding.





Summary

SE-HPLC is a method that is readily available and can be applied using common HPLC system components. Samples can be analyzed under conditions that preserve the structure and function of antigens and antibodies. Immune complex formation can be quantified, and dose-response parameters can be determined that reflect binding affinity (e.g. C50) and maximum antibody reactivity (e.g. maximum CPLX area). Venom:antivenom immune complex formation was analyzed by quantitating changes in SE-HPLC elution profiles of reaction mixtures compared to venom and antivenom control profiles. Three regions were chosen for integration. Region 1 increased due to immune complex formation, Region 2 decreased due to decreased reactant concentrations, and Region 3 did not change during complex formation (non-binding).