SIZE EXCLUSION-HIGH PERFORMANCE LIQUID CHROMATOGRAPHY BINDING ANALYSIS OF CROFAB AND *AGKISTRODON C. CONTORTRIX* VENOM

By

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Chapter I

Introduction

1.1 Forward

The impetus for this study results from activities both personal and professional. Rationally it is easy to see why the *Crotallus atrox* or Western diamondback rattlesnake has the highest rate of envenomation. This snake is actively hunted each year at numerous rattlesnake roundups. More rattlesnakes are kept in captivity by both amateur and professional herpetologists, which could lead to more bites. However, from my personal experience as an outdoor adventure instructor and avid outdoorsman I encounter mostly *Agkistrodon contortrix contortrix* snakes, more commonly known as the Southern copperhead, while in the out of doors.

After doing an initial literature search it was clear that envenomations from rattlesnakes were more substantially researched while envenomations from copperheads were not. It was also clear, with respect to envenomations, the copperhead produced the most reported bites each year, with the exception of the rattlesnake. From phone inquiries to the Oklahoma Poison Control Center, the copperhead in Northeast Oklahoma had the highest incidence of envenomation (personal communication with Ms. Lee McGoodwin). These facts lead me to question why there has not been a more substantial body of work for this type of envenomation.

On closer examination I learned of the newest approved treatment available for snake envenomation. This newly approved antivenin is manufactured by ProTherics Inc, Nashville TN, under the brand name CroFab™. Although it is not manufactured with copperhead venom specifically in mind the ED_{50} for *Agkistrodon contortrix contortrix* is

4mg CroFab™/mg venom, as reported in the product insert (1). In clinical terms, it takes 4mg of CroFab™ for every 1mg of copperhead venom to provide 100% protection for one half of those animals tested from death within 48 hours.

1.2 Background

There are approximately 120 species of snakes that are native to the United States. Of these, about 17% have venom that is harmful to humans. These snakes all fall into the category of pit vipers, with the exception of the coral snake. Venomous snakes have been identified in every state except, Alaska, Maine, and Hawaii (2). Venomous snakes native to Oklahoma include the copperhead, prairie rattlesnake, timber rattlesnake, western cottonmouth, western diamondback rattlesnake, western massasauga rattlesnake, and the pygmy rattlesnake, all of which are in the family *Viperidae* subfamily *Crotaliane.* The subfamily *Crotaliane* includes the genus of *Agkistrodon* (copperhead and cottonmouth), *Crotalus* (rattlesnakes), and *Sistrurus* (massasauga and pygmy rattlesnakes) (3). Each year in the United States there are approximately 7,000 to 8,000 bites by venomous snakes with 5 or 6 related deaths (2,4,5). Deaths typically occur in children or the elderly. Death may also be associated to those individuals who do not seek treatment or if treatment is administered in an insufficient amount of time to reverse the effects of the venom. Most, if not all, bites occur on extremities as a deliberate attempt to handle the snake, or as an unfortunate accident of stepping directly on a snake (2,4,5). Bites occur most often in the period of late spring to early fall when the snakes are most active and people are most often in the out of doors (5). Of the reported snakebites from venomous snakes, 25% are from the copperhead snake. This is more than any other venomous snake, with the exception of the rattlesnake (4,5).

1.3 Venom Components

Snake venom is a specialized cocktail that includes enzymes and toxins which have evolved to aid the snake in the capture and digestion of prey (6). The resulting actions of these enzymes and toxins can have profound effects on both prey animals and humans who are unfortunate enough to be bitten. Of this cocktail, neurotoxins are of particular importance, producing paralysis of skeletal muscles (6). Neurotoxins can be classified according to their site of action within the nervous system, such as pre-synaptic or post-synaptic (2,6). Pre-synaptic toxins have varying actions or activities of phospholipase A_2 and have been identified in the *Viperidae* family as well as others. The action of phospholipase A_2 (PLA_2), which is the most studied component of snake venoms, is associated with the blockage of release of acetylcholine (ACh), the major neurotransmitter in muscle end plates. The inhibition of ACh release at somatic muscle results in paralysis of bulbar and ocular muscles, as well as paralysis of respiratory muscles, which could cause death (2,4,7,8). Post-synaptic neurotoxins also act on muscle, however their effect is to antagonize the nicotinic receptor in skeletal muscle. Post-synaptic neurotoxins have only been identified in two families of venomous snakes, *Elapidae* and *Hydrophiidae*, neither of which are native to the United States (6).

In studies with dogs, phospholipase A_2 is shown to produce a sharp fall in mean arterial blood pressure along with a dramatic rise in pulse pressure. Effects also included, after a lethal dose of venom (0.5-1.0 mg/kg i.v.), changes in EEG or ECG and heart rate, prolonged apnea, with arterial pressure remaining low until respiratory arrest (7). *Agkistrodon* venom PLA_2 (Ag PLA_2) alters mitochondrial respiration and phosphorylation. At low concentrations, Ag PLA_2 increased mitochondrial respiration in

the absence of a phosphate acceptor. At high concentrations, Ag PLA_2 caused severe inhibition of electron transport, and intermediate concentrations of Ag PLA_2 produced a decline in respiration in which ADP acted as an inhibitor (7). High venom concentrations also block axonal conduction in axons of lobster legs and also the giant axons of squid (7). This conduction blockage is attributed to the enzymatic hydrolysis of the acyl bond at the C_2 carbon of cell membrane phospholipids (8). Venom PLA_2 has also been associated with increased cell membrane permeability, especially platelet membranes (7).

1.4 Systemic Effects of Venom

Humans have varying systemic effects as the result of snake envenomation. These effects depend on a number of factors, which include the species of snake, quantity of venom injected, and the time between envenomation and the administration of an appropriate medical treatment (2,4,5). Clinical effects of snake envenomation may range from mild to severe, again depending on the factors described above. However, effects are also dependent on the size of the person bitten with smaller persons showing more profound effects because of receiving a larger dose relative to body size (4,5). Systemic effects include nausea, vomiting, perioral paresthesia, tingling of fingertips and toes, myokymia, lethargy, and general weakness. These symptoms generally occur early after an envenomation (2-5). More severe effects occur after a longer period of time, and often include hypotension, tachypenea, respiratory distress, severe tachycardia, and altered sensorium (2). Components from venoms may also cause an increase in the permeability of capillaries, which results in extravasation of electrolytes, albumin, and red cells into the bite site. This process may also rarely occur in the lungs, myocardium, kidneys,

peritoneum and central nervous system. (2). In more serious envenomations, renal failure may occur from hypotension (2,6,).

Generally the bite of the copperhead snake is thought to be mild or benign in clinical significance. The traditional snakebite severity scoring system used to grade an envenomation is the major factor from which this stems. The scoring system is based on a combination of local and systemic effects, which is weighted toward systemic symptoms and tissue necrosis (4). Table 1 shows the traditional snakebite severity scoring scale (4).

Table 1. Traditional snakebite severity scoring scale

Severity (Grade)	**Manifestations**	**Amount of antivenin recommended (ACP)**
No Envenomation (0)	Local or systemic signs absent	0
Minimal (I)	Local swelling Absence of systemic signs Normal laboratory findings	2-4 vials
Moderate (II)	Swelling extending past bite site (6-12 in) ≥1 systemic sign or symptom Abnormal laboratory findings	5-9 vials
Severe (III)	Marked (>12 in) swelling Tissue loss Multiple or severe systemic symptoms, immediate systemic signs, rapid progression of symptoms	10-15 vials
Very Severe (VI)	Rapid development of local reaction Ecchymosis, necrosis, blebs, blisters Swelling severe enough to obstruct venous or arterial flow, swelling may involve ipsilateral trunk	>15 vials

Copperhead snakebites rarely exhibit these types of symptoms. As a result, the highest grade a copperhead envenomation can receive is II (4,5).

The most significant recent study on copperhead envenomations was conducted in West Virgina during the period from January 1, 1995 to September 30, 1999. In this study ninety-two patients were identified that met the criteria for inclusion in this study (4). Inclusion criteria were that the snake is positively or probably identified as a copperhead. An identification was considered to be positive if the snake was brought in to the emergency department or killed on site and identified by at least one person in addition to the person bitten for example, police, emergency medical service personnel or other adult presumed to be reliable. A local scoring system was developed for this study and is shown in Table 2 (4).

Table 2. Local effect scoring system for copperhead envenomations

Score	0	1	2	3	4
Local Effect					
Pain	None Present	Pain present, no pain management needed	Pain present, APAP or oral NASID sufficient	Pain present, nonopiate* parenteral analgesic required	Pain present, opiate analgesic required for > 24 hr
Swelling	None Present	Confined within 7.5 cm of bite site	Involves greater than or equal to one half of affected extremity	Involves ≤ one half of affected extremity	Extends beyond affected extremity
Ecchymosis	None Present	Confined within 7.5 cm of bite site	Involves greater than or equal to one half of affected extremity	Involves ≤ one half of affected extremity	Extends beyond affected extremity
Time from bite to reach full swelling or ecchymosis	No Progression	> 8 hr	>4 hr ≤ 8 hr	>1hr ≥ 4 hr	≤ 1hr

APAP, acetaminophen: **NSAID**, nonsteriodial anti-inflammatory drug. * For example, ketorolac, butorphanol, nalbuphine, or buprenorphine.

This new scoring system uses different criteria than the traditional snakebite severity grading scale. It is different in the fact that pain plays a major role in scoring. Both scales use a 0-4 scale with a score of 0 having no apparent effects on the patient and 4 being the most severe effects expected. The results in this study show that 1/3 of those patients with copperhead snakebites had a clinically significant local effect with an average score of 3.5 (4). The majority of patients bitten were admitted to the hospital. These results would tend to suggest that the bite of the copperhead is not as benign as once thought.

1.5 Early Use of Fab Fragments

The first antivenin for human use was for the treatment of cobra envenomation (7). The antivenin was prepared using unrefined horse serum and frequently produced anaphylaxis, hypotensive crises, and other numerous unwanted side effects (9,10). In 1954 Wyeth Laboratories, Philadelphia, Pa, introduced Antivenin (*Crotalidae*) Polyvalent (ACP), which until recently, was the only approved treatment available for snake envenomations in the United States. This treatment is often associated with an immediate adverse reaction rate as high as 56% and a delayed adverse reaction of up to 75% (6,5,11). This rate of adverse reaction had practitioners debating on its effectiveness and use. Today most antivenins are either partially purified immunogloublin G (IgG) as produced in the United States or antigen binding fragments (Fab_2) as produced in Europe and other parts of the world (5). Since humans have such adverse reactions to horse sera attempts have been made to produce antivenin from both rabbits and goats. The results from these attempts are encouraging (5,12,13).

The adverse reactions of ACP are well documented, however they are mostly from retrospective studies. In contrast the studies conducted for adverse reactions to a new treatment available, CroFab™, for snake envenomations are more prospective. A rate of incidence of 14.3% (6 out of 42 patients) for acute reactions was reported for CroFab™ in contrast to acute reaction rates for ACP of as high as 56% (9,10). Also reported were the rates of serum sickness for both ACP and the newly approved CroFab™, which was 75% and 16% respectively. It should be noted that CroFab™ has not been shown to produce anaphylaxis. However, experience with CroFab™ is too limited to say whether or not anaphylaxis will occur. (9).

In 1985 Russell *et. al.* demonstrated that an ovine Fab antivenin could be prepared by using affinity chromatography (10). Briefly, gel affinity chromatography was used to identify and purify antibodies to the venoms of four different species of rattlesnake. These antivenins were evaluated for neutralizing deleterious effects such as cytolytic, hemmorhagic, platelet aggregating and lethality. Results from this study were compared to those obtained with ACP using the same techniques. This new antivenin was shown to be more efficacious in neutralizing these deleterious effects while not exhibiting any evidence of producing anaphylaxis or anaphylactic reactions in animals sensitized to horse serum (9,12).

The use of sheep sera to produce Fab fragments is not new, with the first recorded use of Fab fragment antibodies for digoxin (9-11). This antibody was first developed in 1967 as the digoxin immunoassay, and in 1971 digoxin antibodies were first used to treat digoxin toxicity in dogs. The first human patient to receive digoxin specific Fab fragments was in 1976 (9). A Fab fragment that is antigen specific can be produced

relatively quickly and easily for almost any toxin. The process is simple; first an animal is hyperimmunized with the desired antigen (this may be a goat, sheep, rabbit or another animal that is suitable); the animal is then bled, the sera tested for antibodies specific to the antigen, then the antibodies are purified. The antibodies are then digested with papain, which yields two antigen binding fragments (Fab) and one crystalline fragment (Fc) each with a molecular weight of ~50,000 Da. The Fab is purified by gel affinity chromatography. Another type of antibody molecule, which contains 2 Fab fragments (Fab_2), can also be made from the original, also without the Fc, portion by digesting it with pepsin. This molecule can also be effective in neutralizing toxins (1,10,11).

1.6 Treatment Using Fab Fragments

Although treatment for digoxin poisoning has been available since the early 1970's it was not until recent time that a treatment for snake envenomation utilizing a Fab fragment was available. In 1997 two reports were published each claiming to be the first clinical experiences with the use of Fab fragments to treat snake envenomation. One report was published in the Journal of Internal Medicine. This study was used to determine the response of a Fab antivenin against *Viper berus,* which is a viper indigenous to parts of Europe (14). The specific Fab fragment produced to neutralize the effects of *V. berus* envenomation proved to be almost ten times more effective on a weight-to-weight basis as compared to a more traditional equine antivenin (14). This study took place in the period of 1991-1997 with Swedish patients who were responding poorly to traditional treatment. The objective of this study was to determine the clinical efficacy and incidence of adverse reactions associated with the Fab fragment. Results of

this study show that the efficacy of the treatment is comparable to those with the traditional treatment, however at a much smaller dose of antibodies (14).

The second report claiming to be the first clinical experience with a specific Fab fragment for snake envenomation was published in American Journal of Tropical Medicine and Hygiene in 1997 (15). However, this report uses a monospecific antivenin for the treatment of *E. ocellatus* or carpet viper found in Northern Nigeria, which is responsible for greater than 95% of the envenomations in the region (15). For this study a comparison of efficacy between the monospecific Fab antivenin (EchiTab™) and Institute Pasture Serum Africa antivenom (Ipser) was described. Results from this study suggest that 0.5 g (one vial) of the EchiTab™ produce the same response as 2.12 g (four ampules) of Ipser (15).

Regardless of which study is first, results from reports such as these suggest that monospecific ovine Fab fragment antibodies are as effective at neutralizing the effects of snake envenomation as more traditional antivenins of equine origin. Results also suggest that these antibodies are at least as safe, if not more so, than antibodies derived from horse sera. Potential advantages and explanations for this could be because equine versions of antivenins contain large amounts of heavily glycosylated IgG. It is this glycosylated Fc portion of the IgG molecule that leads to greater antigenicity (14).

Unlike intact IgG, Fab fragments only have a single binding site and do not form cross-linked immune complexes. Fab fragments exhibit faster kinetics due to the smaller size, which allows for a larger volume of distribution than conventional antivenins. Finally, the purity of the Fab fragment is much higher due to the affinity chromatography

step in their production. However, the shorter half-life of Fab fragments is a potential disadvantage (14).

1.7 Production of CroFab™

With this knowledge in hand researchers then began a study in which sheep were used to produce antibodies to particular venoms of clinical importance to North America. To produce this antibody/antivenin, four different flocks of sheep were inoculated with one of four types of snake venoms native to the United States. Snakes were chosen with particular criteria in mind: clinical importance in the U.S. and Northern Mexico, geographic range of the snake, genetic dissimilarities of the venom, and cross-antigeneicity with venoms from other clinically important crotalids (1,5,10). Snakes chosen for study were *Crotalus atrox* (Western diamondback), *C. adamanteus* (Eastern diamondback), *C. scutulatus scutulatus* (Mojave rattlesnake) and *Agkistrodon piscivorus* (Eastern cottonmouth) (1,5,10). The sheep flocks were bled and each monospecific immunogloublin was precipitated with Na_2SO_4, redissolved, and then digested with papain (5,6,10). The resultant Fab fragment was then used to determine its effectiveness in mice. In addition to the venoms used to inoculate the sheep, six other crotalid venoms were tested using this antivenin. Results from this study show that the Fab antivenin was significantly more potent than the more traditional ACP, not only against the specific venom used to inoculate the sheep but it also gave relatively good cross-protection from the additional venoms (5,10).

As a comparison, the more traditional ACP is also produced using venoms from four different snakes. However, only two of these snakes are native to North America with the other two native to South America. Also a contrast these four venoms are

injected into a single animal. The horse is then bled and the anti-bodies collected. The antivenin produced from this process not only contains IgG molecules but also horse sera, which leads to the antigenicity of the antivenin.

The technique of affinity chromatography was used in the production and *in vivo* analysis of CroFab™ with emphasis on the ED_{50} for the venom used. CroFab was also tested in mice against venoms not used in its production (1,10). The ED_{50}, for *A. contortrix contortrix,* the venom used in this report, was 4 mg CroFab™/ mg venom tested (1,10).

The hypothesis for this study is there is antivenin venom binding, which can be analyzed using Size Exclusion-High Pressure (performance) Liquid Chromatography (SE-HPLC). This is a technique that has allowed for fast and efficient purification and analysis of peptides based primarily on molecular mass (16-18). Another area where SE-HPLC has proven useful is in the study of antibody-antigen (Ab-Ag) complex formation (19,21). Clinically SE-HPLC has been used to detect, identify and characterize stable Ab-Ag complexes in studies of malaria, insulin autoimmune disease, cancer, and leukemia and lymphoma (19-21). The formation of stable complexes in the case of ACP with *C. atrox* venom has also been described (20,21).

1.8 Use of SE-HPLC

Size exclusion liquid chromatography is a term used to describe the chromatographic separation of molecules based on size or mass (19-21). A small volume of a solution containing the sample is injected and allowed to interact with a column containing a material with pores, which are of comparable size to the molecules to be

separated. The mobile phase is pumped under pressure through the column. The molecule's size determines how much interaction occurs in the column.

The basic principals of SE-HPLC elution profile analysis use are 1) elution time or volume, and 2) peak area or peak height (16,17,22). The elution time or volume is related to the molecular mass of the molecules involved as determined from a series of standards. The injection volume and shape of the molecule also influence the elution time, which could lead to non-ideal elution profiles. The peak area or height is related to the concentration of the components. Commercially available software, such as PeakFit (SPSS, Inc. Chicago, IL) is available for detecting multiple peaks in an elution profile. Other methods for determining the peak height or area have also been described (22).

The fundamental concept of SE-HPLC is that the larger the molecule, the faster it will come out or elute, which makes the analysis of an elution profile relatively straightforward and simple. Other factors, besides the size of the molecule, such as the composition of the eluent or mobile phase, pH of the mobile phase and the composition of the column may also influence at what point in the profile a molecule will elute Modern pre-packed columns contain small particles 15 μm or less which results in larger amounts of surface area with which molecules can interact. They can be safely operated up to a flow rate of 1mL/min, which gives run times of about 20 minutes. Information from the various manufactures can help in determining the proper column needed for the application to produce the desired results.

The formation of a high molecular weigh complex can be determined just by examining an elution profile. The appearance of a peak with elution times that are shorter than the antivenin or venom alone are indicative of complex formation (16,17,22).

It is the appearance of this peak, or the lack of it, which determines if there has indeed been complex formation.

Another consideration when analyzing a SE-HPLC profile is resolution of the profile. Resolution in this case can be defined as the degree of separation of adjacent peaks within an elution profile. Factors that influence resolution could include flow rate of the mobile phase, composition of the mobile phase, pH and the size of the pores in the column. Flow rate of the mobile phase is important in that at a slower rate gives the reactants more time to interact with each other and the column thus increasing resolution. Composition of the mobile phase and pH also can affect resolution by denaturing or precipitating the reactants to the point where no peaks are seen. Resolution may also be affected by pore size. For example, a column with small pores would increase resolution for smaller molecules and decrease resolution for larger ones because of the smaller molecules ability to "get inside" or interact with the internal portion of the column. In contrast, a column with large pores allows for greater resolution of larger molecules and decreases the resolution for smaller ones. Columns are chosen depending on the size of the molecule in question. If a column is chosen with the wrong pore size for the molecule in question, either too small or too large, a non-ideal elution profile will result. Help in choosing the correct column can be obtained from the various manufacturers.

1.9 Purpose of the Study

The goal of this study is to determine if a stable high molecular weight complex is formed between CroFab™ and *A. contortrix contortrix*. This will be accomplished by injecting reaction mixtures a SE-HPLC column and the resulting elution profile analyzed.

To determine if the complex formation is dose dependant various concentrations of venom will be used while keeping the concentration of antivenin constant.

The specific goals of this project are.

1) Determine if a high molecular weight complex is formed.
2) Determine if complex formation is dose dependent.
3) Determine an apparent EC_{50} for the complex. The EC_{50} is defined as the effective concentrations of venom where one half of reactive antivenin is bound to venom.
4) Compare the apparent EC_{50} to the reported ED_{50}.
5) Determine if CroFab™ exhibits any cross reactivity between venoms.

Chapter II

Materials and Methods

2.1 Experimental Design

Venom will be added to a solution of CroFab™, 1mg/mL final concentration, at various concentrations between 0 and 1 mg/mL. This reaction mixture will then be injected into a SE-HPLC column; typically the volume injected will be 20 µL, with a flow rate of 1mL/min. As the reaction mixture elutes it will then pass through a photodiode array detector and the resulting profile will be monitored at 280 nm using the Millennium software (Waters, Milford MA). The resulting elution profiles will be corrected for baseline, and then normalized and integrated as described below.

2.2 Instrumentation

The SE-HPLC system consisted of a Waters (Milford MA) solvent delivery pump, a Waters universal chromatography injector (Model U6K), a Bio-Rad Silect 250-5 (300 x 7.8mm) size-exclusion column, and a Waters 996 photodiode array detector. Column temperature was 25° C (room temperature). Elution buffer was 0.05 M sodium phosphate (pH 7.4) containing 0.05 M sodium chloride. The flow rate was 1 mL/min. Absorbance of the eluate was monitored at 280 nm.

2.3 Antivenin, Venom, and Standards

The Antivenin, CroFab™, was a generous gift from Suzanne Ward, director of product management for Protherics. Antivenin was rehydrated with elution buffer according to the instructions from the manufacturer, and then further purified using the SE-HPLC system. Approximately 500 µL of a stock solution of antivenin (10 mg/mL) was injected into the SE-HPLC system and the major peak containing the Fab fragment

SigmaPlot (SPSS, Inc., Chicago, IL). Each set of elution profiles was also compared and adjusted for sample size and concentration.

2.6 Method of Analysis Using Elution Profiles

Analysis of mixtures containing antivenin and venom were based on methods described for antibody-antigen interactions using SE-HPLC (19,21). These methods were modified to fit this study. The basis of the analysis of reaction mixtures is based on the assumption that either the concentration of reactants and products in the mixtures are proportional to either peak areas or peak heights from the chromatograms. This assumption has been used to determine antivenin-venom binding that occurs based on equations derived from the law of mass action (19,21).

The relationship of the elution profile area to component area is generalized by

$$T = A + B + C, \qquad [1]$$

where T is the total profile area and A, B and C are unreacted antivenin, unreacted venom and venom-antivenin complex area, respectively. The relationship of total profile area to concentration of components can be generalized by,

$$T = \alpha \cdot (A_0) + \beta \cdot (B_0), \qquad [2]$$

where (A_0) and (B_0) are mg/mL antivenin and venom respectively, α and β are proportionality constants determined from a series of control mixtures and represent the slope of the linear equation for unreacted antivenin and venom controls. Profile areas were normalized to 1 mg/mL A_0 by,

$$T_{norm} = \alpha + \beta\,(B_0)/(A_0) \qquad [3]$$

where T_{norm} is the normalized profile area. Normalized profiles were produced by,

$$P_{norm} = (P_{obs} / T_{obs}) \cdot T_{norm} \qquad [4]$$

where P_{norm} is the normalized profile, P_{obs} is the observed profile and T_{obs} is the total area of the observed profile. This procedure is useful for normalizing data using other concentrations of reactants and for combining information from different sets of reaction mixtures.

2.7 Normalization of Elution Profiles

Variables, which may affect the elution profile, may include injector malfunction or not injecting exactly 20 μL, detector response, and experimental error. Using the procedure described above normalizes all profiles. The result of the calculations produces a profile that is normalized to 1mg/mL antivenin. Figure 1 is a representative elution profile of the same reaction mixture before and after normalization. The conditions for the profile are 1mg/mL CroFab™ and 1 mg/mL copperhead venom. Plot A in figure 1 are injections of the same reaction mixture ran at different times before any normalization has been performed. The figure shows the profiles are not identical, with differences in the peak heights. Plot B in figure 1 is the same reaction mixture profiles after normalization has been performed. The plots are much closer to identical. Normalization not only takes into account the variables described above, it also allows the reaction mixtures from different experiments to be combined and compared on an equal basis.

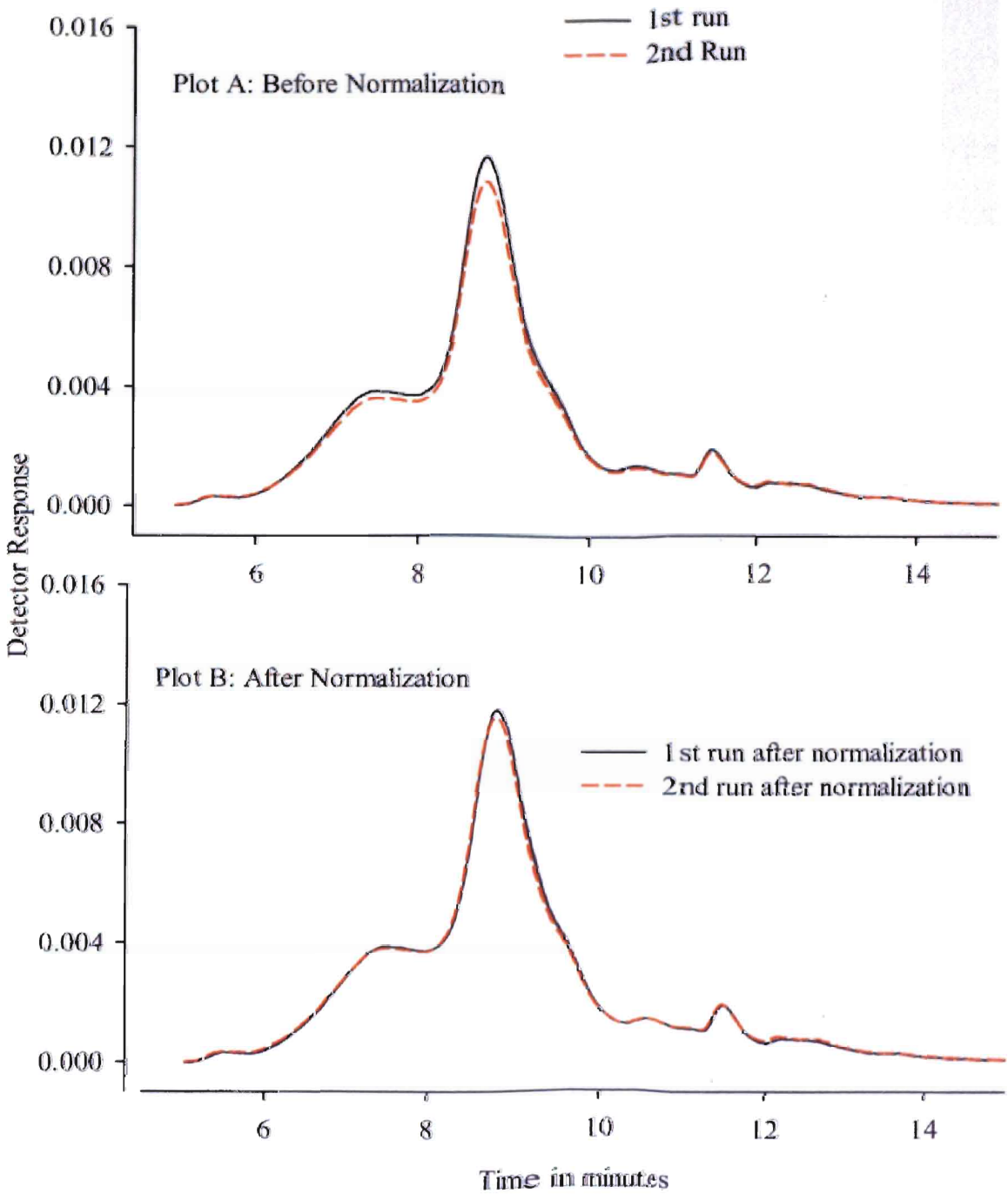

Figure 1. Representative elution profiles of a 1 mg/mL antivenin and 1 mg/mL venom reaction mixture. Plot A is the 1st and 2nd run of the same reaction mixture before any normalization procedure. Plot B is the same elution profiles after the normalization procedure has been performed.

2.8 Integration of Profiles

Integration of elution profiles is relatively straightforward and easy to perform. On examination of the profiles of the standards it can be determined which of the reactant molecules would elute first, in this case the antivenin, and any molecule detected before the expected time may be any complex formed. The resultant profiles are then examined and time points are chosen for the integration step. The selection of time points is critical to the process. If inappropriate time points are chosen some complex may be missed and its area underestimated or too much area may be assigned to the complex. This is also true for the antivenin with not enough or too much area assigned. For the integration step in this study integration times were, 5 min to 7.5 min for complex formation, and 8 min to 10 min for the antivenin. The areas related to these time points allows for optimal area of each component to be assigned.

2.9 Synthesis of Null Profiles

In order to determine if indeed there was a high molecular weight complex formed or if there were any changes in profiles, null profiles were needed. Null profiles are profiles expected if no reaction were to occur between antivenin and venom. Null profiles can be synthesized by adding the profiles of each venom and antivenin controls.

2.10 Analysis of Cross Reactivity

In 1998 Stevens *et al* (23) described a method in which SE-HPLC was used for epitope mapping. In this method an antigen is incubated with two antibodies separately and then with the same antibodies together. The resulting elution profiles are then compared with that of a synthetic null profile produced by adding the profiles of reaction mixtures containing the antibody and antigen with that of the third component, which has

been independently chromatographed. To evaluate the profiles for epitope specificity a difference or delta profile is then produced. This profile is the result of subtracting the synthetic null profile from that of the reaction mixture that contains all components.

A positive first peak in the delta profile indicates the increased formation of high molecular weight peaks. From this increased complex formation it can be said that the antibody recognizes spatially or structurally different epitopes on the surface of the antigen. In contrast, a negative first peak indicates a reduction in the formation of high molecular weight complexes. A reduction in high molecular weight complexes reveals binding specificities for overlapping or identical sites (23). This method of epitope mapping was modified to fit this study. In contrast to epitope mapping the procedure was used to determine if the binding of antivenin to venom exhibited any cross reactivity between venoms, or if the antivenin has any residual reactivity for one venom after binding with another venom.

To determine if there is any cross reactivity between venom used in the manufacturing of CroFab™ and copperhead venom, a cross reactivity study was performed. This was accomplished by initially incubating a reaction mixture containing either copperhead or rattlesnake venom with CroFab™ for 1 hour at 37^0 C. After the initial incubation period the reaction mixture was divided in half. An additional aliquot of elution buffer was added to one reaction mixture and an additional aliquot of the opposite venom was added to the other. Final concentrations were 0.5-mg/mL for the venom and 1.0 mg/mL for CroFab™. Samples of reaction mixtures, typically 20 µL, were injected into the column and the eluent monitored at 280nm. The information gathered was used to determine if the antivenin exhibited cross-reactivity between

venoms. Additionally, the data was used to determine if binding was independent in nature.

Chapter III

Results

3.1 Controls

Representative elution profiles of CroFab™, *A. contortrix contotrix*, and *C. atrox* are shown in figures 2, 3 and, 4, respectively. These chromatograms represent the controls at various concentrations used in this study (Fig 5). Proportionality constants α (antivenin), β_1 (copperhead), and β_2 (rattlesnake), were calculated using data from figure 5 using the linear equation for the slope of the line for total area of the chromatograms. Those proportionality constants were then used to normalize the profiles of each mixture to 1 mg/mL of CroFab™ by using the procedure described under Materials and Methods.

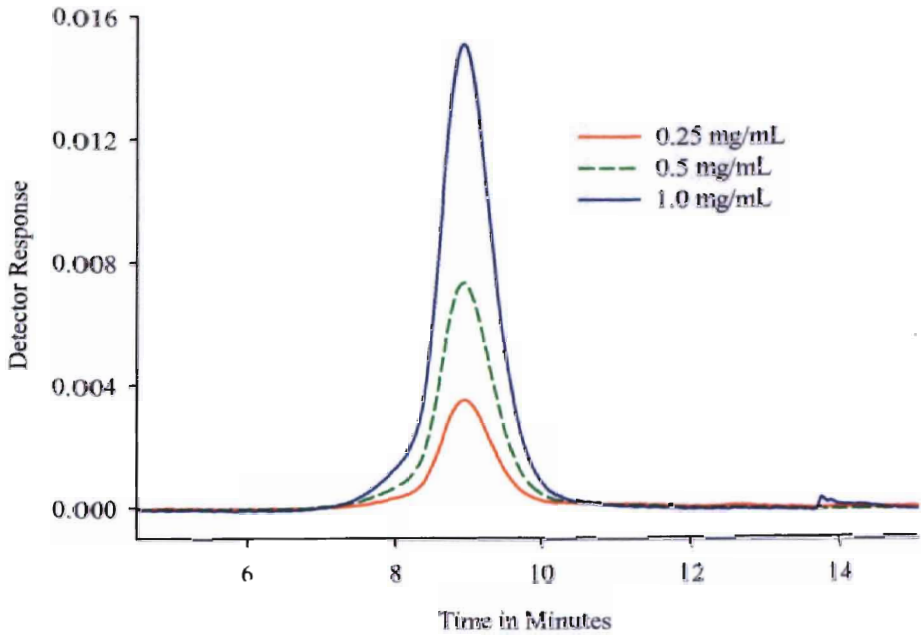

Figure 2. Representative CroFab™ elution profiles. Elution profiles were obtained as described in Materials and Methods.

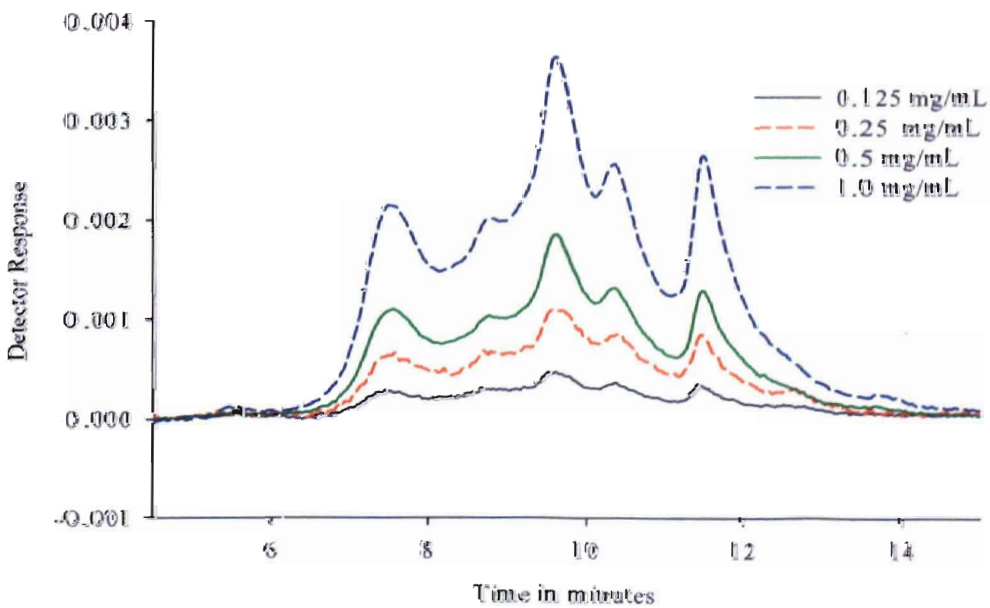

Figure 3. Representative *A c. contortrix* elution profiles. Elution profiles were obtained as described in Materials and Methods.

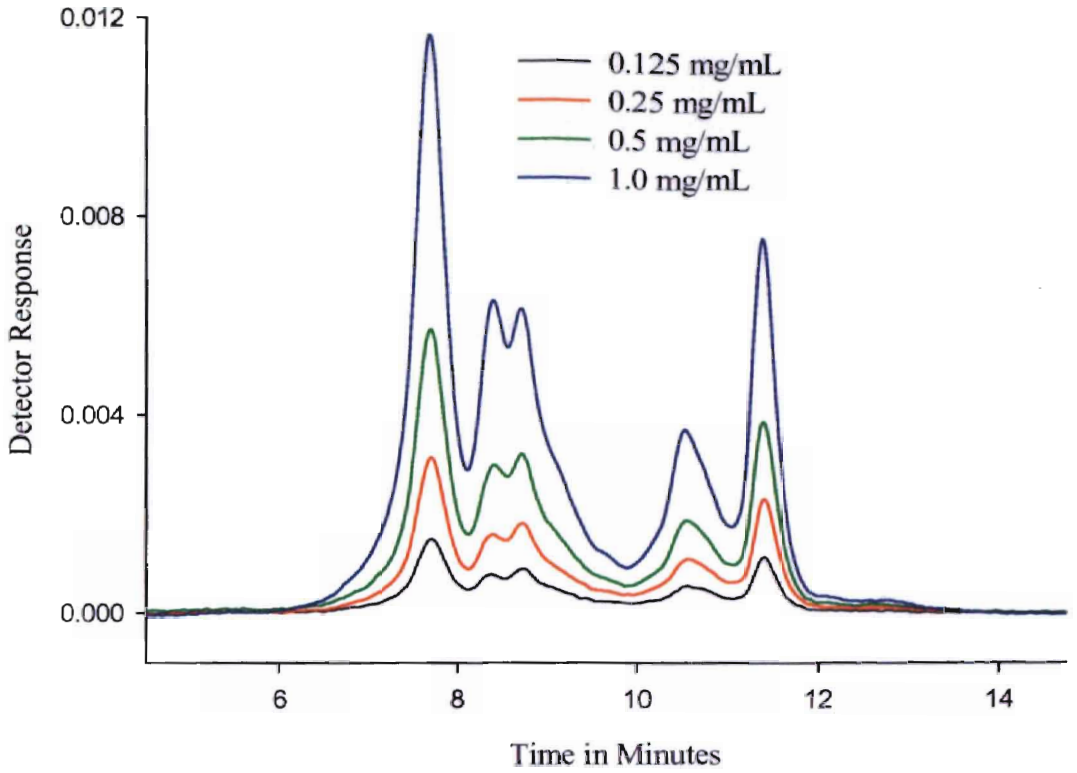

Figure 4. Representative *C. atrox* elution profiles. Elution profiles were obtained as described in Materials and Methods.

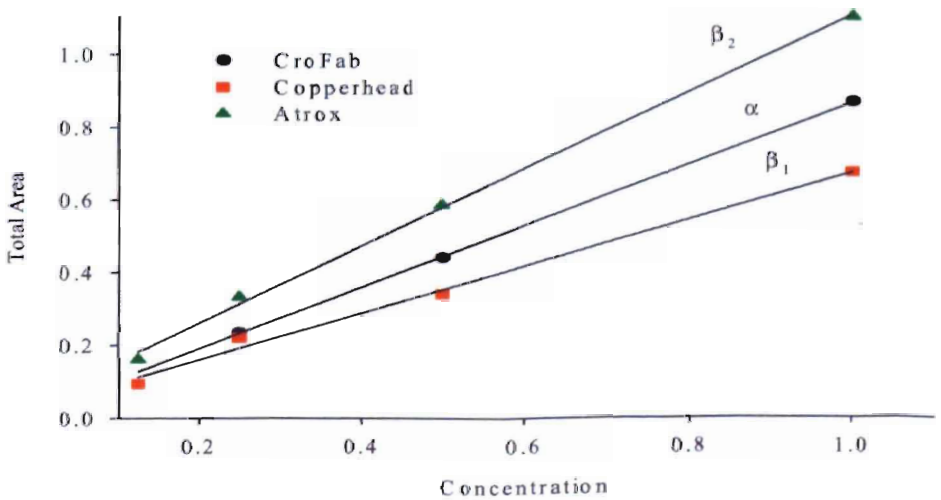

Figure 5. Total profile areas of controls. The slope of the linear equation is used to determine proportionality constants α, β_1, and β_2.

3.2 CroFab™ vs. Copperhead

Figure 6 shows a series of normalized synthetic null profiles of mixtures of CroFab™ and copperhead venom. Plot A of figure 6 represents the elution profile of a 1mg/mL CroFab™ control, a 1 mg/mL copperhead venom control, and a synthetic null profile of a reaction mixture of antivenin and copperhead venom at 1mg/mL. Figure 6 plot B is the synthetic null profiles for each concentration of copperhead venom used for controls and is representative of no reaction taking place. Regions of the elution profile where integration was performed are also indicated on the plot.

Figure 7 is normalized elution profiles of reaction mixtures of antivenin and copperhead venom. Plot A in figure 7 represents a 1mg/mL CroFab™ control and a reaction mixture containing 1mg/mL CroFab™ and 1mg/mL copperhead venom. Figure 7 plot B is representative of reaction mixtures of CroFab™ and copperhead at various concentrations. Evidence of complex formation is demonstrated by the formation of a high molecular weight complex and the decrease in the antivenin and venom component peaks with increasing venom concentration. Figure 7 plot B also suggests that complex formation is dose dependant with complex area increasing with increasing venom concentration. Two major peaks designated cplx (complex) and cfab (CroFab™) are seen. Areas of the elution profile where integration was performed are indicated on the plot.

Figure 8 shows the integrated peak areas for the areas chosen as a function of increasing venom concentration. Included are null complex, reaction complex (cplx), CroFab™ (cfab), the null CroFab™ area, and the total area of the profile. The graph is another indication of complex formation as well as confirming the dose response

relationship of antivenin to venom. The plot also demonstrates how different data sets can be combined and provides a graphical means of evaluating relative reaction component and complex peak areas. From the fit of the data to a hyperbolic function of

$$Cplx = Cplx_{area} \bullet (Ven_0) / (EC_{50} + (Ven_0)) \qquad [5]$$

it was determined that the apparent EC_{50} for copperhead venom was 0.05 mg venom / mg CroFab™ ($R^2 > .99$, $P < 0.001$, $SE < 0.01$).

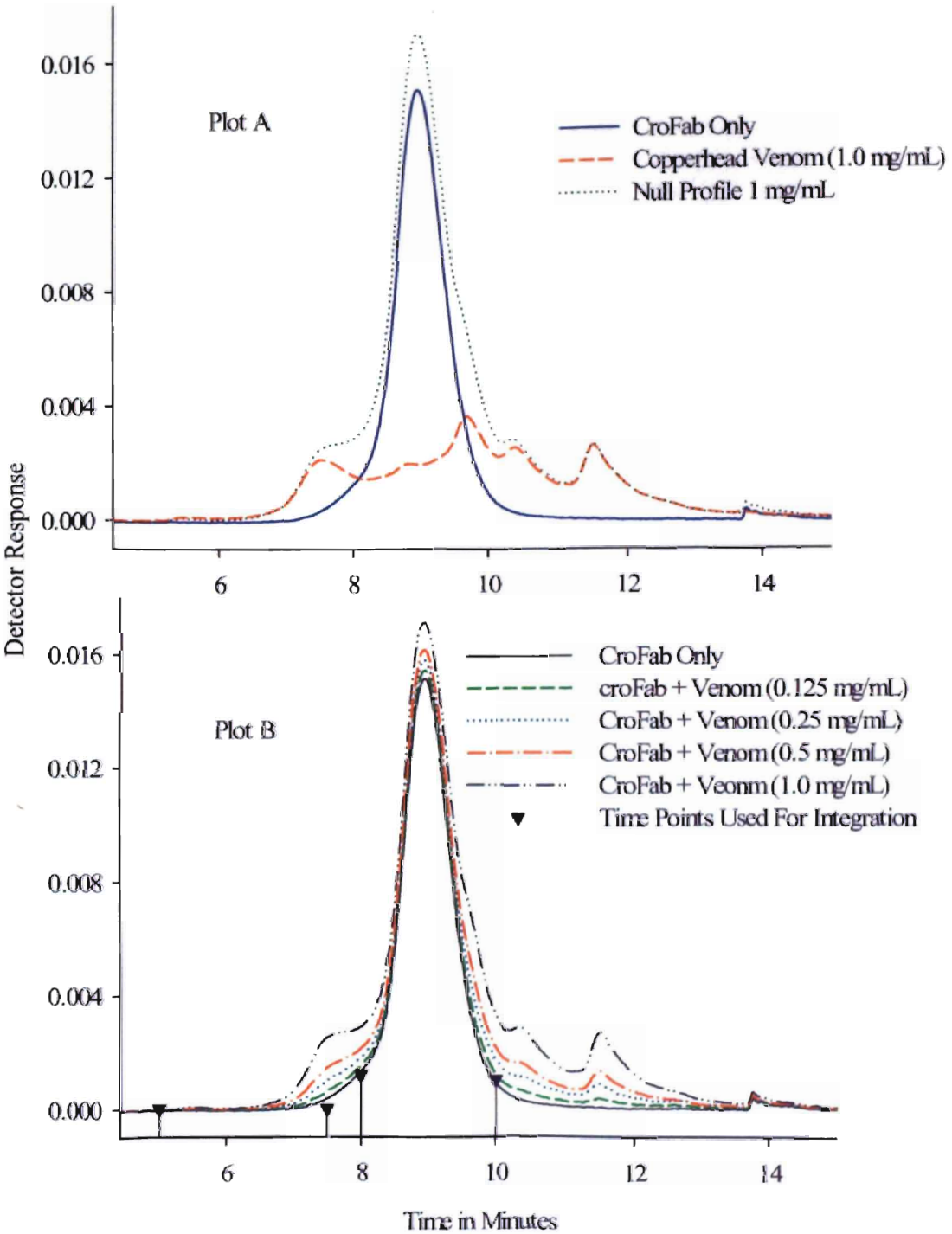

Figure 6. Representative synthetic Null profiles. Plot A is 1 mg/mL CroFab™ a 1 mg/mL venom control and a synthetic null profile of a 1 mg/mL antivenin-copperhead venom mixture. Plot B are the profiles expected if there were no reactions or complexes formed over the full range of concentrations used.

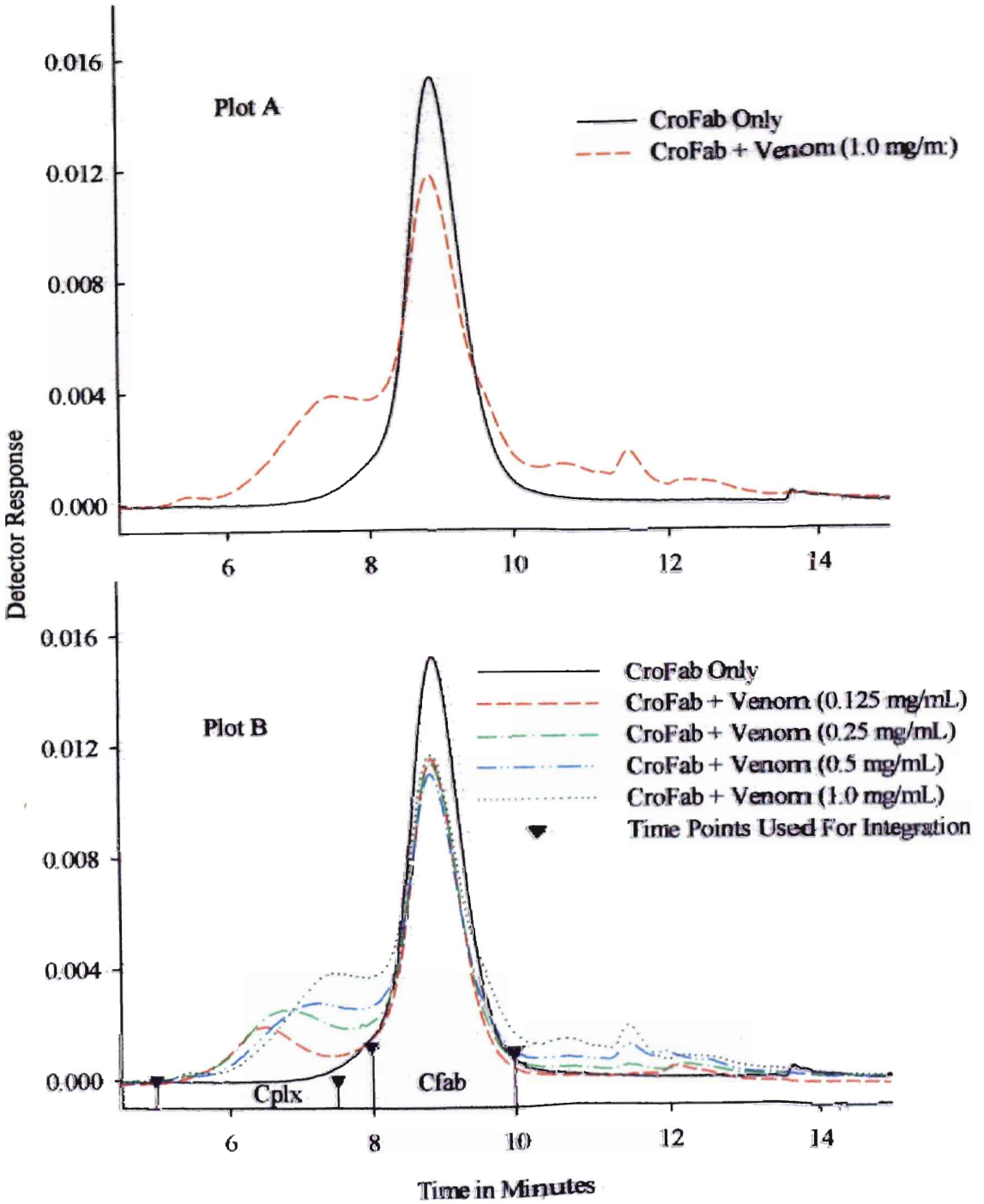

Figure 7. Representative elution profiles of reaction mixtures. Plot A is the elution profiles of CroFab™ alone and a reaction mixture of 1 mg/mL copperhead venom. Plot B is representative of all reaction mixtures after normalization. Two major regions are seen and designated Cplx (complex) and Cfab (CroFab™). Drop lines indicate time points where integration was performed.

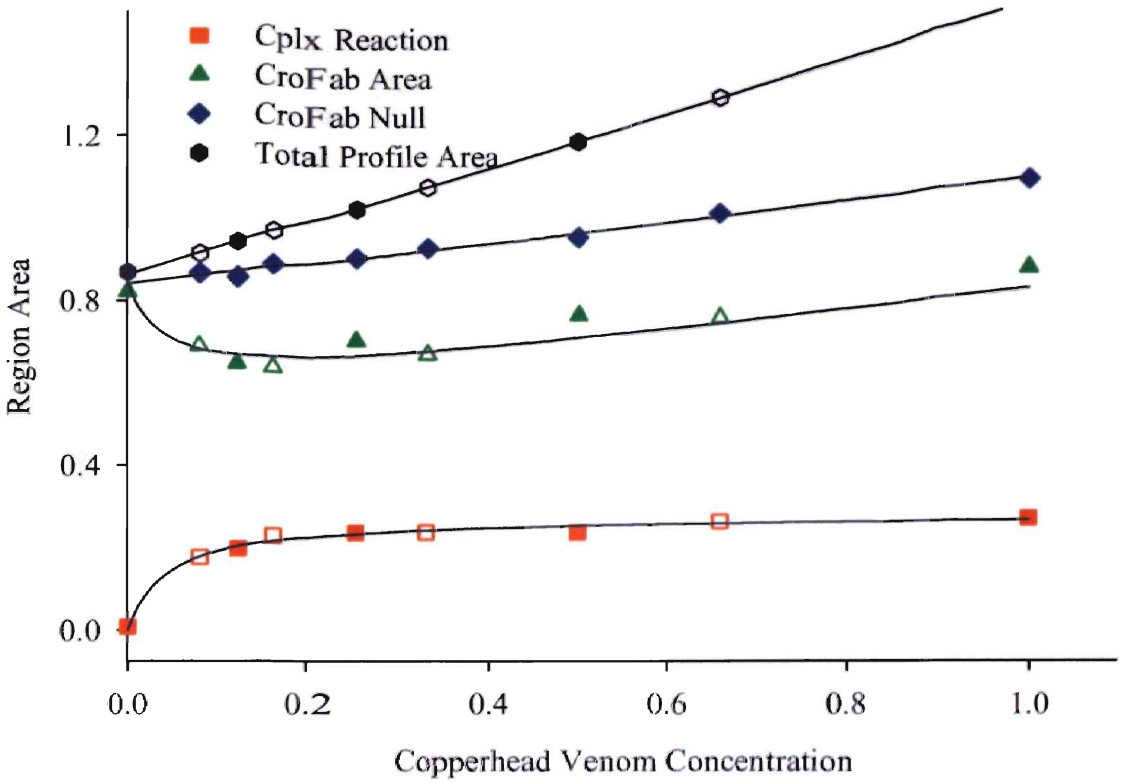

Figure 8. Relative peak areas normalized to 1 mg/mL CroFab™ at various concentrations of copperhead venom. Data points represent the mean of duplicate runs from 2 experiments conducted on different days. Included are relative peak areas expected if no reaction were to take place (Null) and data from reaction mixtures. Open symbols are data from 10/16/02, closed symbols are data from 12/16/02.

3.3 CroFab™ vs. Rattlesnake

Because CroFab™ is not manufactured with copperhead venom a comparison was performed with a venom that is used in it's manufacturing. *C. atrox* venom was the one chosen for this comparison.

The elution profiles shown in figure 9 show a series of synthetic null profiles of mixtures of CroFab™ and rattlesnake venom. Plot A of figure 9 represents a 1 mg/mL CroFab™ control, a 1 mg/mL rattlesnake venom control, and a synthetic null profile of a reaction mixture between antivenin and rattlesnake venom at 1 mg/mL. The elution profiles shown in plot B of figure 9 are the synthetic null profiles of 1 mg/mL antivenin and various concentrations of rattlesnake venom. Regions where integration was performed are also indicated on this plot. These plots represent the expected elution profile if no reaction were to take place between reactants.

The elution profiles in figure 10 are profiles of normalized reaction mixtures. Plot A represents the reaction mixture of antivenin and 1mg/mL rattlesnake venom. Plot B is the elution profiles seen with various concentrations of rattlesnake venom while holding the antivenin constant at 1 mg/mL. Integration time points are also included in this figure.

As with copperhead venom evidence of complex formation is demonstrated by the formation of high molecular weight complexes and the decrease in the antivenin and venom component peaks with increasing venom concentration. Figure 10 plot B also suggests complex formation is dose dependant with complex area increasing with increasing venom concentration.

For analysis of profiles, regions were chosen comparable to those chosen for copperhead venom, with complex (cplx), venom (ven) and CroFab™ (cfab) regions identified. The raw data obtained was normalized to 1mg/mL CroFab™ by using the procedure previously described.

Figure 11 shows the integrated profile areas for regions chosen as a function of increasing venom concentration. Included are null cplx, cplx, null cfab, and total profile area. The graph is further evidence of complex formation as well as confirming the dose-response relationship. From the fit of the data to a hyperbolic function, (equation [5]), an apparent EC_{50} for rattlesnake venom was determined and is 0.04 mg rattlesnake venom / mg CroFab™ (R^2>.98, p<0.005, SE<0.01).

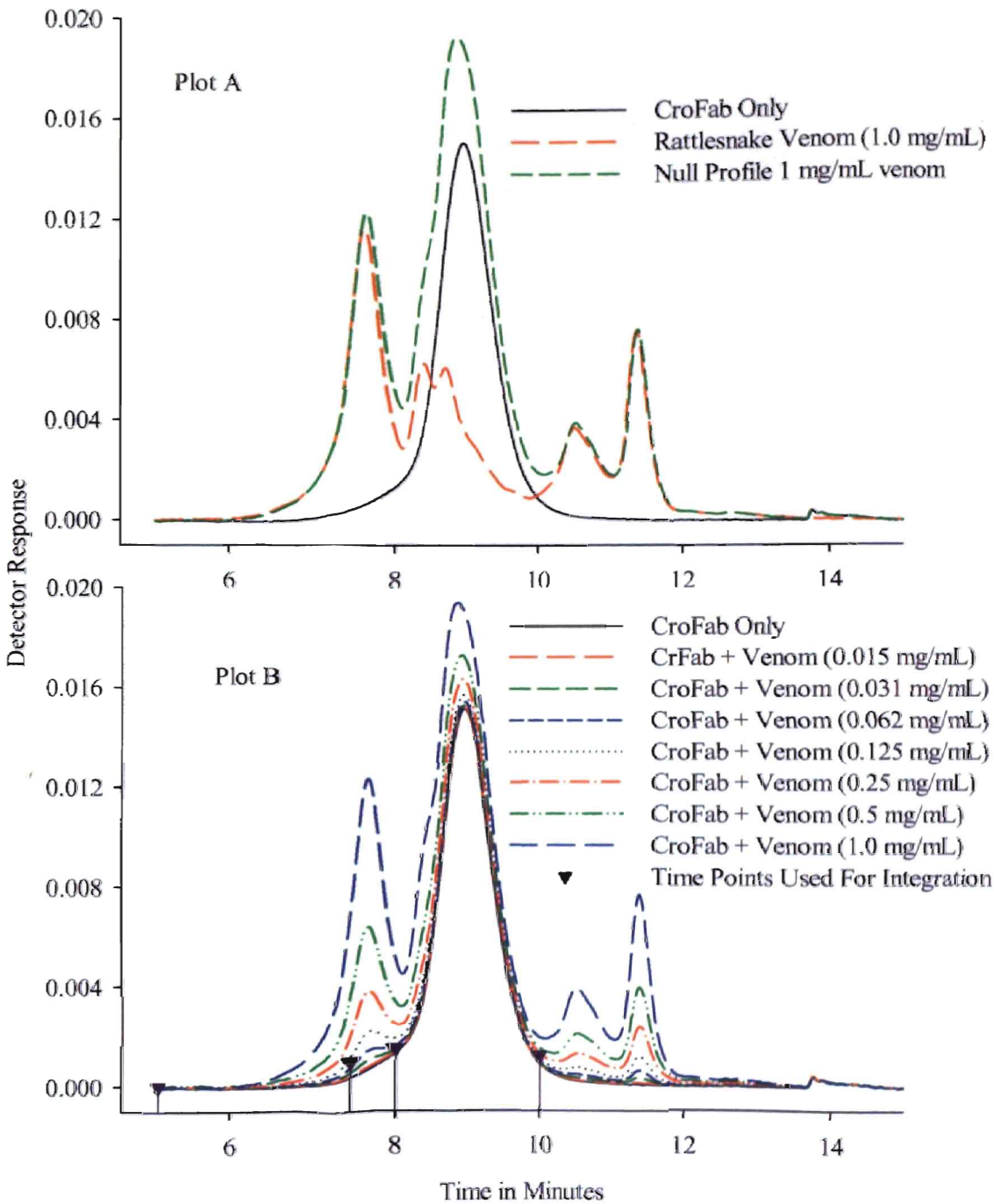

Figure 9. Representative synthetic null profiles of CroFab™ vs. *C. atrox* venom. Plot A represents CroFab™ alone, a 1 mg/mL venom control and a synthetic null profile of a 1 mg/mL antivenin-rattlesnake venom mixture. Plot B are the profiles expected if there were no reactions or complex formed over the full range of concentrations used.

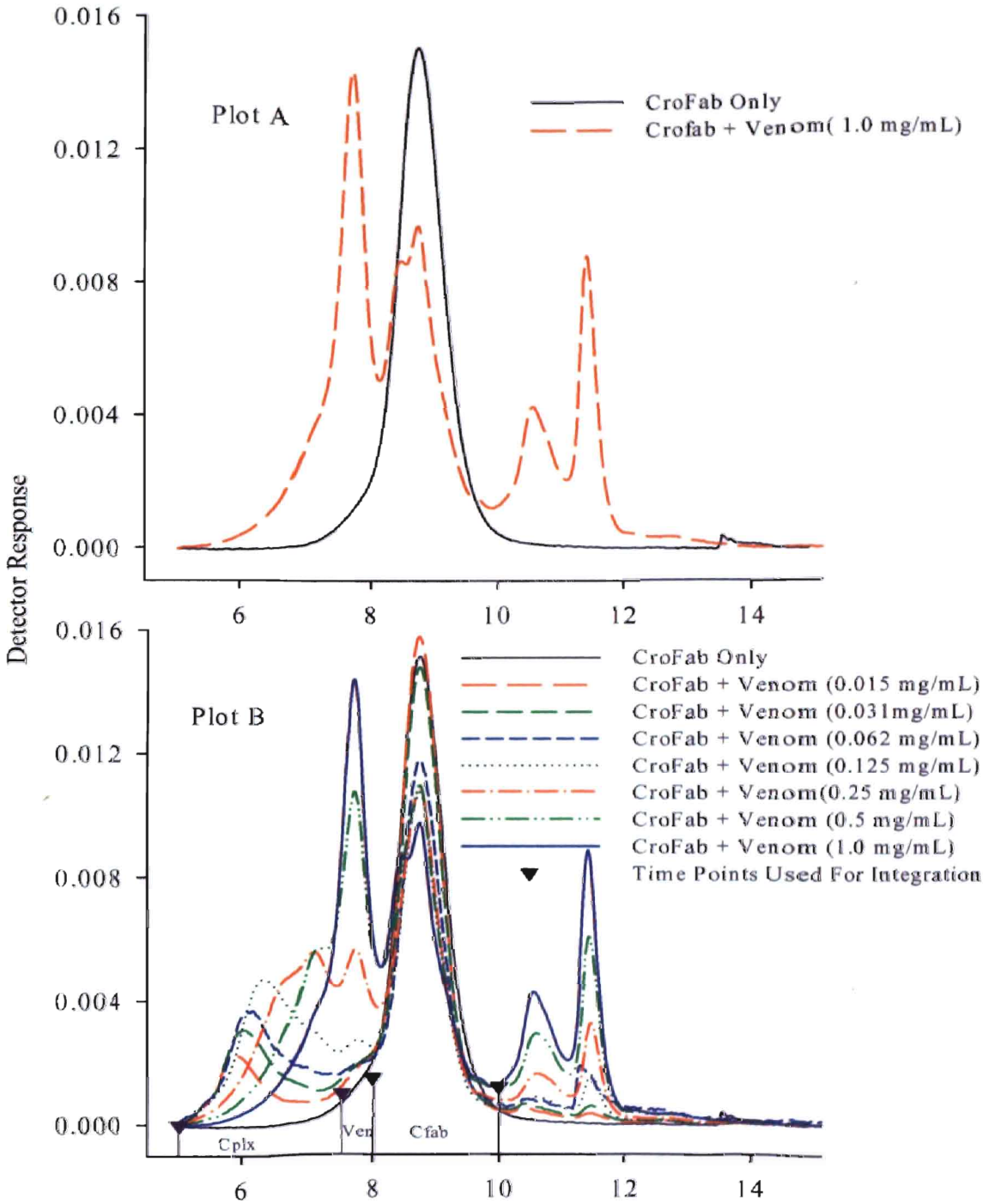

Figure 10. Representative elution profiles of CroFab™ vs. *C. atrox* venom reaction mixtures. Plot A is the profile of CroFab™ alone and a 1 mg/mL venom reaction mixture. Plot B is the elution profiles of reaction mixtures at varying venom concentrations. Plot B also shows the time points where integration was performed.

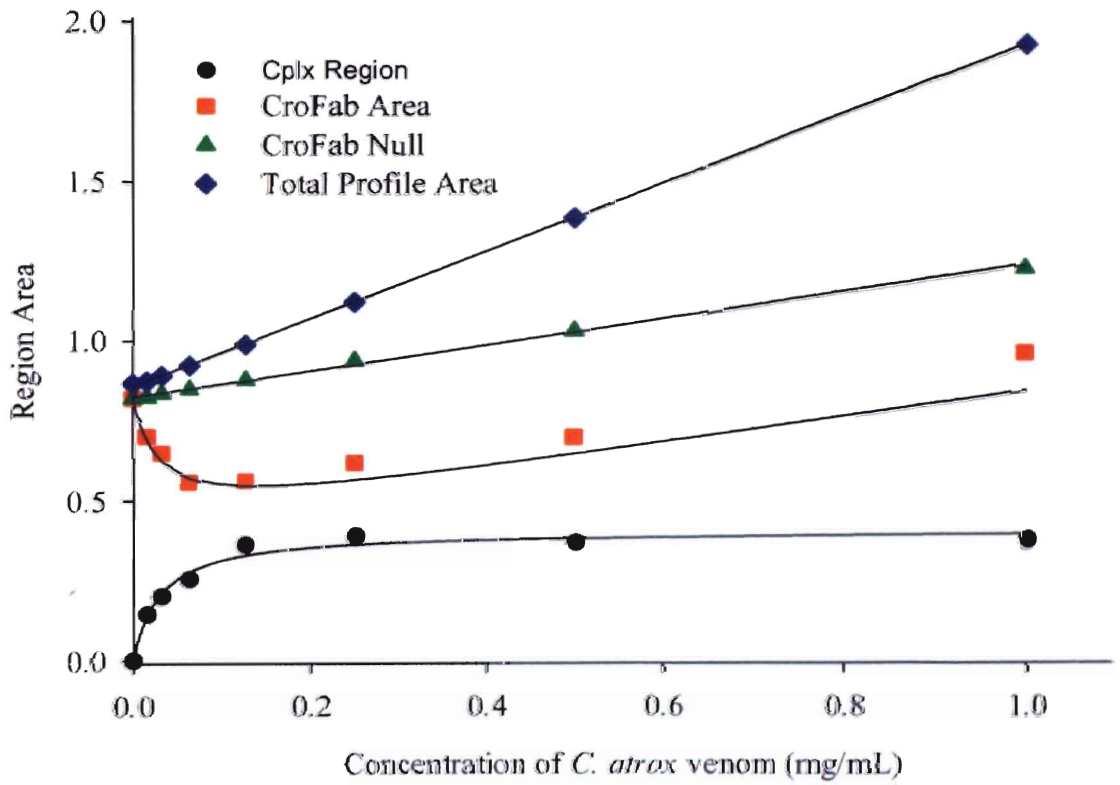

Figure 11. Relative peak areas normalized to 1 mg/mL CroFab™ at various concentrations of *C. atrox* venom. Data points represent the mean of an experiment ran in duplicate. Included are relative peak areas expected if no reaction were to take place (null) and data from reaction mixtures.

3.5 Analysis of Cross Reactivity

Analysis of cross reactivity was used to determine if copperhead venom and rattlesnake venom bound to CroFab™ independently (non competitively) or competed for binding to CroFab™. The procedure described in Stevens, *et al* (23) for epitope mapping was modified and used. This procedure uses difference, or delta, profiles to determine competitive or noncompetitive binding of antigen with antibodies. Null profiles are synthesized by adding the profile of a reaction mixture with the profile of a control that had been previously chromatographed (23). Subtracting the null profile from that of the reaction profile generates difference profiles. The difference profiles are then used to determine any changes in reaction mixtures. Positive peaks in the complex region and negative peaks in the CroFab™ and venom region indicated binding has occurred.

Control mixtures containing either CroFab™, copperhead venom or rattlesnake venom alone were incubated for 1 hr at 37^0 C. Reaction mixtures containing either copperhead venom and antivenin or rattlesnake venom and antivenin were incubated for 1 hr at 37^0 C, after which the reaction mixture was divided in half. An additional aliquot of either elution buffer or the opposite venom was added and then incubated for an additional hour.

Raw data was collected and normalized using the procedure previously described. Figure 12 plot A represents the null, reaction mixture, difference profile and a 1 mg/mL copperhead venom, for the reaction mixture of antivenin with copperhead venom. The null for this profile was constructed by adding together the profiles of CroFab™ and copperhead venom controls. The difference profile in this plot is indicative of complex

formation with an increase of complex area and a decrease of antivenin area. A leading peak in the difference profile that is positive indicates that additional binding has occurred after the initial reaction has taken place.

Plot B figure 12 represents the reaction mixture of antivenin and rattlesnake venom with copperhead venom added after initial incubation. The null was constructed by adding the profile of the reaction mixture for antivenin and rattlesnake venom with that of the copperhead venom control. Evidence of complex formation is seen in the difference profile of increasing complex area and decreasing antivenin area.

Figure 13 plot A represents the reaction mixture of CroFab™ with rattlesnake venom. Included in this plot are profiles for the null, the reaction mixture, a copperhead venom control and the difference profile. Plot B of figure 13 shows the profiles of the reaction mixture for antivenin and copperhead venom with rattlesnake venom added after initial incubation. Both plots are indicative of complex formation with increasing complex area and decreasing antivenin area.

Figure 14 compares difference profiles. Plot A compares difference profiles for copperhead venom, the red plot is copperhead only and the blue plot is after the introduction of rattlesnake venom. Plot B is rattlesnake venom with and without copperhead venom. In both instances there seems to be a decrease in complex area, which may indicate that binding of venom to antivenin is not completely independent.

Figure 15 compares the difference profiles of an observed reaction mixture containing mixture containing CroFab™ + copperhead + rattlesnake to that of one expected if binding is completely independent. The difference profiles indicate that the binding of antivenin to venom is not completely independent. The difference between these two

plots indicates that approximately 15 – 20 % of binding is competitive. Analysis of the data for this portion of the study indicates that the order in which the venoms are introduced does not matter with the profiles being almost identical. All profiles were adjusted for injection volume, flow rate and detector response.

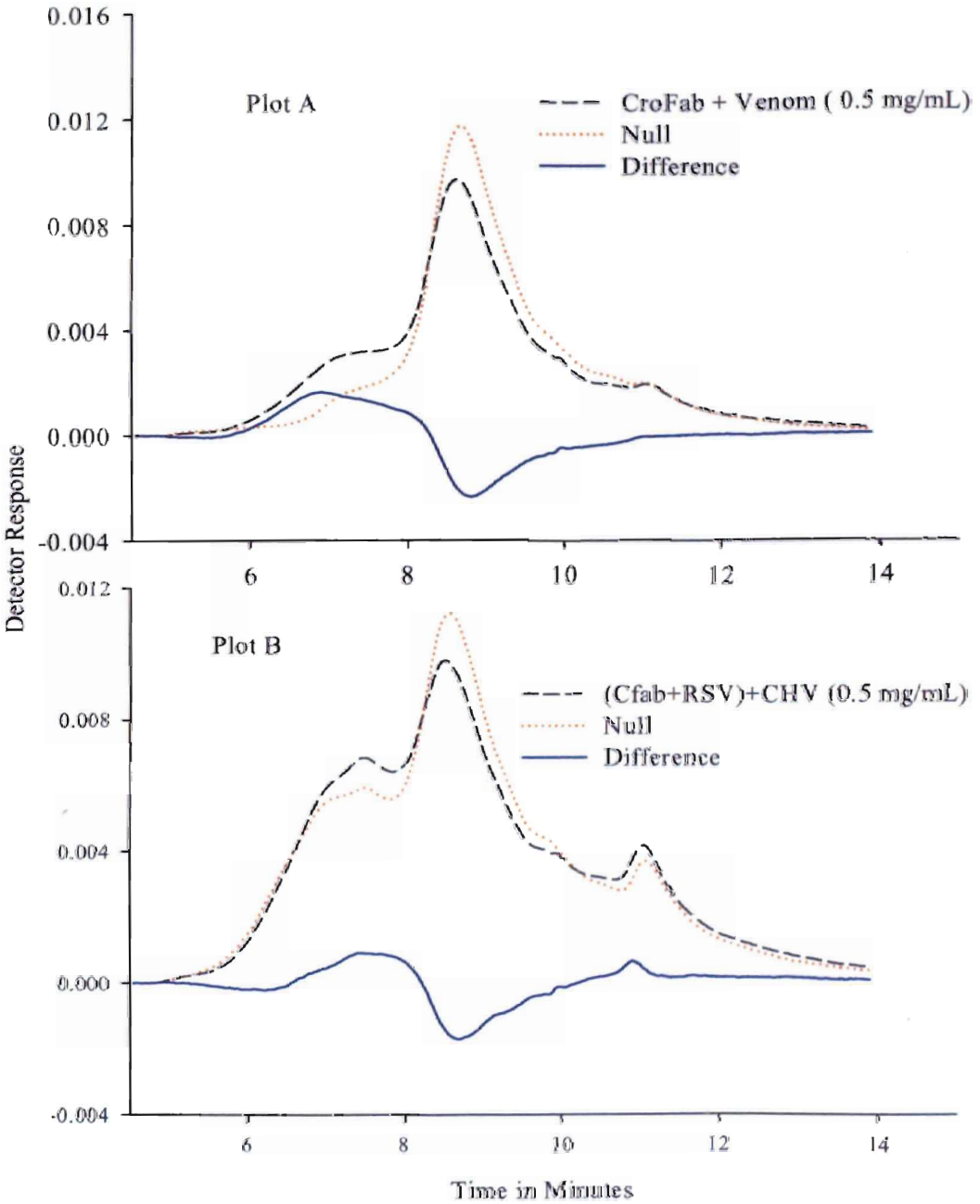

Figure 12. Representative elution profiles for reaction mixtures of CroFab™ and copperhead venom. Plot A contains the reaction mixture for CroFab™ and copperhead venom alone, the null profile and the difference profile for the two. Plot B is the reaction mixture of CroFab™ and rattlesnake venom with copperhead venom added after initial incubation. Also included are the null and difference profiles. Final concentrations were 1 mg/ml CroFab™ and 0.5 mg/mL venom.

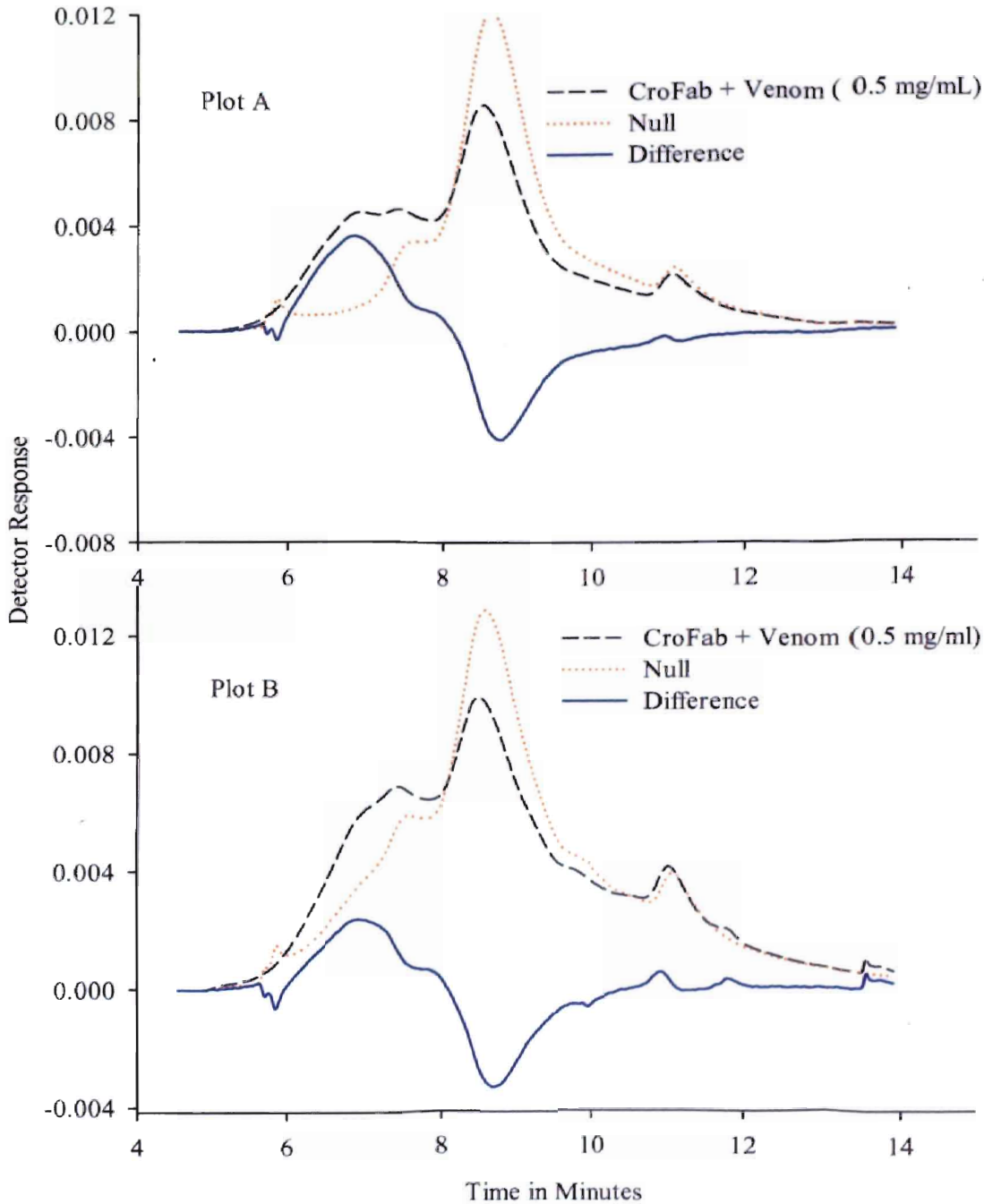

Figure 13. Representative elution profiles for reaction mixtures of CroFab™ and rattlesnake venom. Plot A contains the profiles for the reaction mixture of CroFab™ and rattlesnake alone, the null profile and the difference profile for the two. Plot B is the reaction mixture of CroFab™ and copperhead venom with rattlesnake added after the initial incubation. Also included are the null and difference profiles. Final concentrations were 1 mg/mL CroFab™ and 0.5 mg/mL venom.

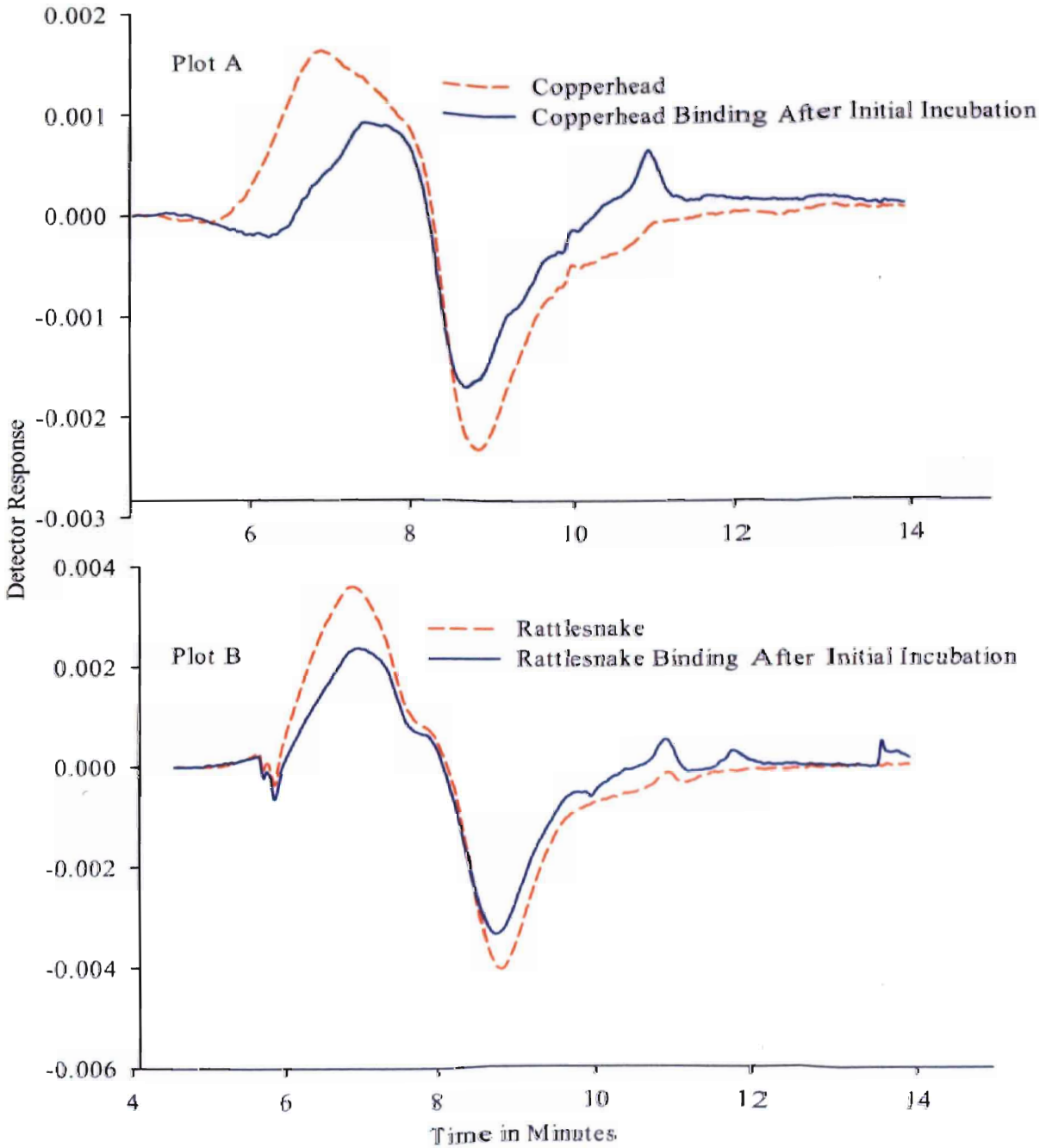

Figure 14. Comparison of difference profiles for copperhead and rattlesnake venom. Plot A is the difference profiles for copperhead venom. The red plot is the difference profile for copperhead venom alone and the blue plot is the difference for copperhead venom after initial binding of rattlesnake venom with antivenin. Plot B is the difference profile for rattlesnake venom. The red plot is the difference for rattlesnake venom alone. The blue plot is the difference after initial binding of copperhead venom with antivenin.

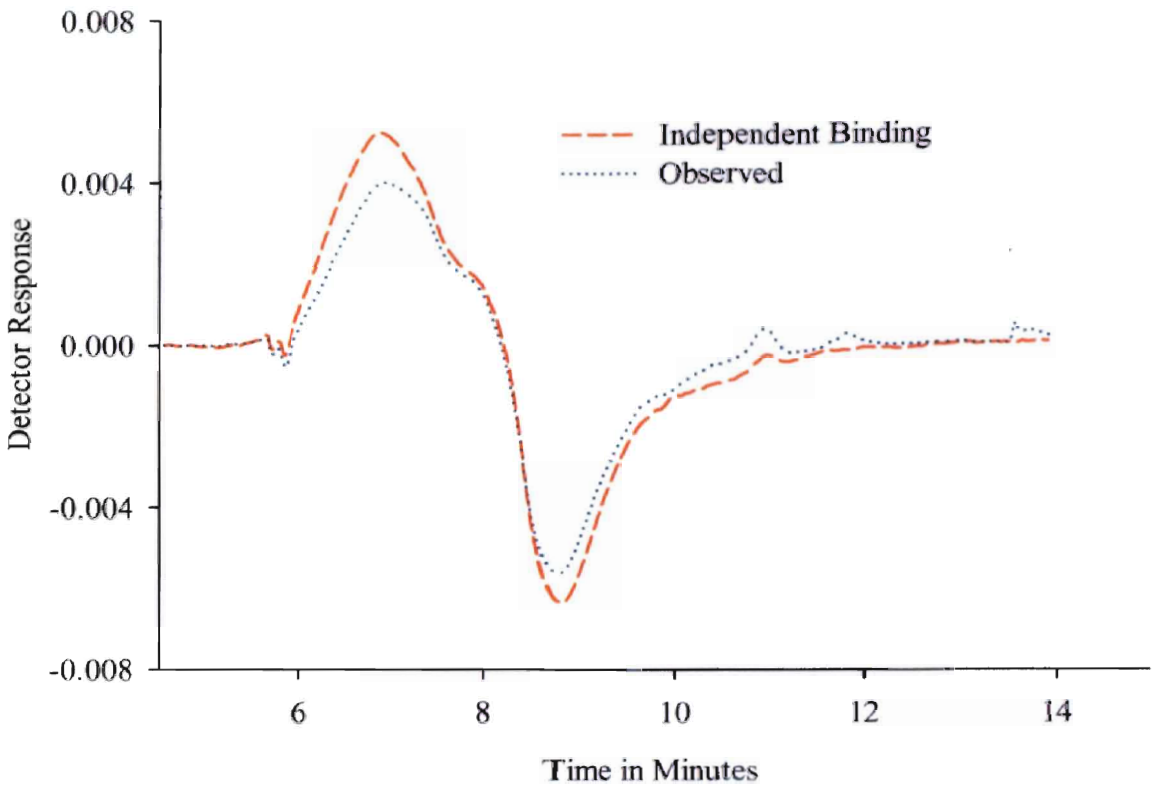

Figure 15. Comparison of independent binding versus binding observed. If binding were completely independent the curve expected would be that of the red plot. The blue plot is the observed binding difference profile.

Chapter IV

DISCUSSION

4.1 Current Treatments

At present there are only two approved treatments for *Crotalinae* envenomation in the United States, the more traditional ACP and the new CroFab™. Although the indications for the administration of treatment for snake envenomation have not been well defined the literature indicates that most common indication appear to be a progressive venom injury (4,5,9). Indications such as progressive swelling, clinically important coagulation abnormality, or systemic effects such as hypotension, or altered mental status lead practitioners to administer treatment. The expected result of treatment is the resolution of those symptoms associated with the envenomation and the prevention of further injury as a result of envenomation.

Each treatment has advantages as well as disadvantages. Perhaps the most important of theses is cost. The more traditional ACP has been the mainstay of treatment in the United States for decades and as such the cost is relatively low, around $650 for a kit that contains all components needed for treatment. However, the cost associated with CroFab™ is somewhat higher, upwards of $1,000 per vial. The course of treatment indicated by the product insert for CroFab™ is an initial dose of 4 to 6 vials. If initial control is not achieved the re-administration of the initial dose is indicated. After initial control is achieved then additional 2 vial doses every 6 hours of up to 18 hours or an additional 6 vials (1). In a worst-case scenario that could mean up to 18 vials of antivenin administered which could result in a cost nearing $20,000, just for antivenin. However, the main advantage associated with CroFab™ is safety. Although experience

with CroFab™ is limited it has not been shown to cause anaphylaxis, whereas anaphylactic reactions associated with the use of ACP are well documented (4,9).

There is one other viable treatment option for copperhead envenomation. This option stresses a conservative approach, or one in which is no antivenin is administered. However, this does not mean a physician should not evaluate the bite just that no antivenin is administered. In one 12-year study, fifty-five patients of copperhead envenomation were successfully treated conservatively without the use of antivenin or surgical excision (5). Conservative treatments in this study were administered to all patients and included i.v. fluids, tetanus toxoid, steroids and antibiotics. Each individual was evaluated and treated by a general surgeon since surgical excision is also an option if treatment was not progressing as desired (5).

This method is a result of the copperhead venom being considered as clinically benign. As a result of the 12-year study the author recommends that copperhead snakebites be categorized separately from other snake envenomations and a conservative approach taken with respect to treatment. However, the author of the study also goes on to state that should the patient deteriorate at any time a more traditional treatment should be instituted (5).

The unpredictable nature of snakebites often makes assessment and management difficult. The literature can be difficult to interpret because of different methods of evaluation, for example the different scoring charts for snake bite severity (Tables 1 and 2). Also there are no uniform guidelines as to the amount of antivenin that should be administered. As a result some clinicians choose to treat snakebites without the use of

antivenin while others only use antivenin in moderate to severe envenomations. Regardless of the choice, antivenin is still the standard therapy for most snakebites.

4.2 Use of Ovine Fab Fragments

As previously discussed, the use of ovine sera to produce Fab fragment is not a new technique. The introduction of an ovine Fab fragment in 1971 for treatment of digoxin poisoning in dogs. With the first use of a digoxin specific Fab fragment treatment for humans documented in 1976 (9-13). In another report a patient was successfully treated for tricyclic-antidepressant poisoning using an antibody produced with ovine sera (26). With the introduction in the United States of CroFab™ clinicians have a choice as to which antivenin to use.

Although today there is only one ovine Fab antivenin treatment approved for envenomation of snakes native to North America around the world there are others that are available, mostly for envenomation for snakes in Africa and Europe. One of which is EchiTab™, manufactured by Therapeutic Antibodies LTD, of Atlanta Georgia. This antivenin is produced for treatment of *Echis ocellatus* or carpet viper envenomation. The carpet viper is a snake native to Africa, responsible for 95% of serious envenomations in Nigeria, with 15 deaths attributed to it each year (15). Another example is ViperaTab®, which is also manufactured by the maker of CroFab™, is being marked in Europe for *Vipera berus*, or the European common adder, envenomation. An example of an antivenin made from ovine Fab fragments, which is in the testing stage, is one for the *Walterinnesa aegyptia* or desert black cobra native to Saudi Arabia. Although this antivenin is not yet in production the results from the initial study are promising. In testing this new antivenin completely neutralized PLA_2 activity (24).

From the lethality studies done in mice, CroFab™ is approximately 5 times more potent than ACP, or in clinical terms, only one-fifth as much antivenin is needed to produce the same degree of protection based on the data (1,9,11). The use of purified Fab should also negate the immunogenic and allergic properties of intact IgG and its corresponding Fc portion. Also other sheep serum components and antibodies that are non-specific to the venom are eliminated by the purification step in the production of the antivenin. Additionally clinical experience with the antidigitalis drug, digoxin, indicates the considerable safety of sheep Fab products (11).

Fundamentally it can be seen that CroFab™ would be more effective with less adverse side effects than the more traditional ACP. First, venoms from four snakes native to North America are used in its production, while ACP is produced from two North American and two Central and South American snakes. Second, CroFab™ is a fragment of an IgG molecule with the Fc portion removed while ACP is an undigested intact IgG molecule (11). Lastly, CroFab™ is produced using affinity chromatography which removes any non-reactive components versus ACP production may contain non-reactive IgG molecules. These factors combined should lead to the more widespread use of CroFab™ in the future.

4.3 Relevance for use of SE-HPLC

SE-HPLC has enabled researchers to carry out peptide purification and analysis of peptides up to one hundred times faster than more conventional low-pressure methods such as gel filtration (25). The main applications of the technique in relationship to the study of peptides are, purification and analysis of purity, estimation of molecular weight,

and the study of protein-protein interactions (25). It is protein-protein interactions that are relevant to this study.

Since the association of polypeptides with other molecules causes an increase in size, these interactions can be measured by SE-HPLC. This technique also can provide information on the stoichiometry of binding, rate kinetics associated with binding and also the distribution of different complexes in the reaction mixture (23,26). Concentration dependant complex formation of multiple forms of human Cu/Zn oxide dismutase, murine monoclonal antibody complexes, as well as two forms of human IgE complexes have been characterized by SE-HPLC (16). Also reported are the formations of stable venom-antivenin complexes using SE-HPLC (17,18).

The technique of SE-HPLC has also been used in the identification and localization of epitopes on proteins of a known sequence and structure (23). Stevens *et.al* demonstrated that a reactive Fab fragment in the presence of an antigen with an epitope that has a distinct location will result in the formation of a complex that is of a higher molecular weight than that of the antigen and a reactive IgG. Also if epitopes overlap or undergo a conformational change which results in the loss of an epitope the molecular weight of the complex will be lower than expected for independent binding sites (23). The technique has also be useful in the determination of the kinetics of binding for Fab fragments to antigen epitopes (23,26).

Antibody-Antigen interaction in mixtures containing reactive and nonreactive components using SE-HPLC was modified to fit this study (17). The use of SE-HPLC has several favorable characteristics, the reaction occurs in solution, which may limit the effect of artifacts associated with protein-protein interactions occurring on the surface of

a gel. Conditions such as pH and temperature are comparable to those types of reactions occurring *in vivo*, and results that are produced are easy to interpret and easily done without the use or raidonucleotides with only the instrumentation found in any average chromatography laboratory (17).

These factors lead to the choice of SE-HPLC in this study. For this study to be successful a stable high molecular weight complex formation was required and is indeed seen. Figures 8 and 11 illustrate the dose-response, concentration dependant appearance of a high molecular weight complex with corresponding decrease in antivenin peak area. Figures 7 and 10 show profiles of reaction mixtures of antivenin and venom. These figures are evidence which indicates the formation of a high molecular weight complexes with the corresponding decrease in both antivenin and venom components. These figures also relate to objectives 1 and 2, which are 1) Determine if a high molecular weight complex is formed and 2) If a complex is formed determine if it is dose dependant.

4.4 Analysis Techniques

In this study, as with any chromatography elution profile study, variables influence the performance of the instrumentation. Factors such as pH, detector response, and injector malfunction may all lead to non-ideal elution profiles. As such, techniques must be employed to combat these variables. For this study analysis of the elution profiles tended to be very straightforward and mechanistically easy. However, some variables were introduced. Because of the sensitivity and inherent background noise associated with the photodiode array the first item corrected for was baseline by using software from PeakFit (SPSS, Inc., Chicago, IL). Once this was accomplished the next task involved normalization. Normalization allows for comparison of elution profiles

from the same reaction mixture ran at different times. Theoretically the same reaction mixture will have the exact elution profile provided all variables are equal. However, rarely are all variables equal. To account for the differences in profiles, areas were normalized to 1mg/mL CroFab™. This normalization allows for the comparison of elution profiles of the same reaction mixture injected at different times. Normalization also allows profiles of different concentrations within the data sets to be compared on an equal basis. Figure 1 relates to the normalization process. In plot A of this figure it can be seen that even after baseline correction the profiles are not identical. Plot B of Figure 1 shows that after normalization the elution profiles are much more standardized. The same process was applied to every elution profile, the information combined and a more ideal elution profile was the result. These new elution profiles make the determination necessary to answer objectives 1 and 2 much easier.

The final step of analysis for this study is integration. For this process time points were chosen along the X-axis of an elution profile. Integration is much more straightforward than normalization, however the process is just as crucial to the analysis of the profile. If the wrong time points are chosen then a problem with area associated to each portion of the profile may occur. Portions of the profile may have too much area assigned to it or not enough. If the region assigned for integration is wrong then the results will be skewed.

The scope of this study allowed for the use of a microcomputer for data acquisition and features of software programs such as Excel to perform integration. Regions for integration of chromatograms for this study were determined by looking at the elution profiles. When a peak is first detected that is where integration begins and

integration stops at the point the peak ends. This continues until the peaks of interest are all integrated. It is the areas under these peaks, which have been normalized and integrated, that are of importance to this study.

4.5 Relevance of Data Obtained

The ED_{50} for CroFab™ in mice studies is 4 mg CroFab™/mg copperhead venom (1,10). For this study to be successful, the formation of a stable antivenin-venom complex was needed and is indeed seen. For reaction mixtures, concentration dependant changes in elution profiles (Figures 7,10) indicate the formation of stable complexes. Objective 3 asks that an apparent EC_{50} for CroFab™ be established. From the fit of a hyperbolic function (Eq. 5) to the data for complex area in Figures 9 and 11 an apparent EC_{50} was determined for both copperhead and rattlesnake venom. The apparent EC_{50} for copperhead venom is 0.05mg venom/mg CroFab™ (R^2>.99, $p<0.001$, SE<0.01), and for rattlesnake venom is 0.04mg/mL (R^2>.98, $p<0.005$, SE<0.01).

To compare the apparent EC_{50} to that of the reported ED_{50} a linear relationship between EC_{50} and EC_{100} and between ED_{50} and ED_{100} was assumed. The EC_{50} is defined as the effective concentration of venom where one half of reactive antivenin is bound to venom (mg venom / mg CroFab™). The ED_{50} (mg CroFab™ / mg venom) is defined as the concentration of antivenin within a mixture of antivenin and venom that will protect 50% of the animals tested from death within a given time period (e.g. 48 hr). As previously stated the apparent EC_{50} for copperhead venom is 0.05 mg venom / mg CroFab™. The reported ED_{50} for copperhead venom is 3.6 mg CroFab / mg venom (11). By using the assumption of linearity, the EC_{100} and ED_{100} for copperhead venom can be calculated as 2 x EC_{50} and 2 x ED_{50} respectively. By using this calculation the EC_{100} for

copperhead venom is 0.10 mg venom / mg CroFab™ and the ED_{100} is 7.2 mg CroFab™ / mg venom. For comparison of the EC_{100} to the ED_{100} using the same units (i.e. mg venom / mg CroFab™) the reciprocal of ED_{100} is used ($1/ED_{100}$) which gives 0.14 mg venom / mg CroFab™ compared to the EC_{100} of 0.10 mg venom / mg CroFab™. The reported ED_{50} for rattlesnake venom is 5.2 mg CroFab™ / mg venom (11) and the apparent EC_{50} is 0.04 mg rattlesnake venom / mg CroFab™. The reciprocal of ED_{100} for rattlesnake venom (ED_{100} = 10.4) is 0.097 mg rattlesnake venom / mg CroFab™ which compares to the EC_{100} of 0.08 mg rattlesnake venom / mg CroFab.

These results indicate that this method may be valuable in providing an estimate of ED_{50} using *in vitro* estimates of EC_{50}. This estimate would be valuable as a starting point in the determination of the actual *in vivo* ED_{50}. This method, if used, could result in a significant reduction in costs associated with live animal testing

Objective 5 asks if the antivenin exhibits any cross reactivity between the venoms tested. The profiles in Figures 12-15 are used to answer this question. To determine if any cross reactivity exists one needs to look at the comparison of delta profiles in Figure 15. If there was no cross reactivity between the venoms and CroFab™ the delta profile would be that of independent binding as shown in Figure 15. However, Based on the data in figure 15 approximately 15-20% of the binding is not independent.

The intent of this study was to examine the binding of CroFab™ with that of copperhead venom. As such the results indicate that approximately 30% of CroFab™ is reactive and at the ED_{50} 90% of the reactive CroFab™ is bound. Although CroFab™ is not produced with copperhead venom, the published data reports good cross reactivity

with it (1,10,11). The data shown in this report, for example the evidence of complex formation the apparent EC_{50} and delta profiles would tend to support this earlier data.

4.6 Summary

Antibody-antigen interaction in mixtures containing reactive and nonreactive components using SE-HPLC (4) was modified to fit this study. This method has several favorable characteristics. The reaction occurs in solution, which may limit the number of artifacts associated with antigen-antibody interactions occurring on a surface (19). Conditions, such as pH and temperature, are comparable to those types of reactions occurring *in vivo*. Results produced are easy to interpret and are easily done without the use of raidonucleotides with only the instrumentation found in the average chromatography laboratory (17). Further analysis using isolated venom components or venoms from other snakes used in the production of CroFab™ could provide more insight on the fraction of reactivity and EC_{50}, or even cross-reactivity between other venoms or venom components. The method of SE-HPLC is applicable for any system in which stable complexes are formed. It is the detection of stable complexes that may be relevant to avidity.

Although results from this *in vitro* study show the total reactive CroFab™ is only 30% this could be related to effects of antivenin and venom interactions *in vivo*. These interactions *in vivo* may involve other interactions not directly associated with complex formation or the formation of less stable complexes not detectable by SE-HPLC. Dissociation of less stable complexes may result in peak trailing or changes in peak shape and elution time of both antivenin and venom. Kinetic rate contributions to simulated SE-HPLC elution profiles from protein-protein interactions have been described (14,20).

Small dissociation rates (e.g. $\leq 10^{-2}$ s^{-1}) would tend to favor separation of bound and free components during the chromatographic run, as would as higher flow rates (17). Further, ultrafiltration or affinity chromatography may be used to separate antivenin-venom complexes and unbound antivenin from both high affinity and low affinity unbound antigen in the reaction mixtures (17). This method could provide an estimate of total bound and free antivenin.

The method of SE-HPLC is simple, fast, and easily interpretable it is applicable to a variety of systems where protein-protein interactions, which produce stable complexes, are studied. Analysis of chromatographic data may provide information about reactive an non-reactive components, the magnitude of antibody-antigen interactions including estimates of binding parameters, identity of antigenic components, and the relationship between antibody-antigen interactions and avidity (17).

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VITA 2

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