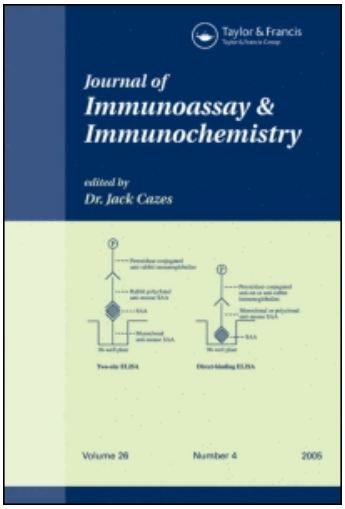

Journal of Immunoassay and Immunochemistry
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597271

Comparison Between Therapeutic Antitoxin F(ab)$_2$ Fractionated with Ammonium Sulfate and Caprylic Acid

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To cite this Article Redwan, El-Rashdy M.(2006) 'Comparison Between Therapeutic Antitoxin F(ab)$_2$ Fractionated with Ammonium Sulfate and Caprylic Acid', Journal of Immunoassay and Immunochemistry, 27: 4, 319 — 329
To link to this Article: DOI: 10.1080/15321810600861993
URL: http://dx.doi.org/10.1080/15321810600861993

Journal of Immunoassay & Immunochemistry, 27: 319–329, 2006

ISSN 1532-1819 print/1532-4230 online
DOI: 10.1080/15321810600861993

Comparison Between Therapeutic Antitoxin $F(ab)_2$ Fractionated with Ammonium Sulfate and Caprylic Acid

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Abstract: To date, animal derived therapeutic antibodies represent the best and only choice source of antitoxins, especially in developing countries. Furthermore, this industry needs to develop a production protocol to achieve safer products. Recently, several laboratories changed their production protocol from ammonium sulfate (AS) protocol to caprylic acid (CA) fractionation. Our results showed that using the CA protocol leads to improvement in the product quality, as assessed by the albumin and protein content decrease (from 4.75 to 3.54 g/dL and 0.64 to 0.18 g/dL, respectively), which yielded a purer antitoxin product. The $F(ab)_2$ protein aggregate formation and turbidity have been significantly reduced, 4.60 versus 2.55 and 0.046 versus 0.021 ($p < 0.01$), respectively. However, the anti-complementary activity was also reduced, from 42 to 33. The total IgG content was higher in CA fractionated products than AS materials. The endotoxin content was worrisome in some $F(ab)_2$ products.

Keywords: Horse $F(ab)_2$ antibody, Fractionation protocols, Ammonium sulfate, Caprylic Acid, Impurities, Anti complementary

INTRODUCTION

Antivenom is derived from antibodies of immunized animals, the rates of reactions appear to vary with the species of antibody origin, the extent of

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pepsin digestion, the presence of molecular aggregates, and the total protein content of the product. It has been presumed that most acute reactions relate to the extent of complement activation from Fc receptor binding, with improvements in quality having largely resulted from enhancements in antivenom processing.[1–4] In response, the World Health Organization (WHO) held its first antivenom workshop in more than 20 years to discuss the global supply and quality of antivenoms. However, perhaps more importantly, a growing community of physicians from the regions with the greatest snakebite burden has become more active in seeking solutions to at least some part of this ancient problem. However, the recent WHO workshop called for a re-examination of old assumptions concerning such reactions and, consequently, what constitutes "best practice" for antivenom manufacture and administration.[5]

Most protocols for antivenom production are based on ammonium sulfate precipitation, with or without pepsin digestion.[6] These procedures have undergone little innovation during the last decades, making the introduction of new methodologies that would give products of higher quality and priority. Several new alternatives have been proposed, such as affinity and ion-exchange chromatography, either alone or in combination with saline fractionation and enzyme digestion. Although highly adequate in terms of quality of the product, these methods are difficult to adapt to production laboratories. Thus, there is a need to develop new procedures for antivenom production which would combine technical as well as economical advantages. Rojas et al.[6] has found that CA-fractionated antivenoms have superior production time, albumin/globulin ratio, turbidity, protein aggregates, electrophoretic pattern, and neutralizing potency against several activity of *Bothrops asper* venom.

Gene et al.[7] showed that AS-fractionated antivenom induced plasma coagulation under some experimental conditions, probably owing to presence of traces of thrombin in this preparation. Antivenom fractionated with caprylic acid did not have this problem, probably owing to its higher purity. Moreover, CA-fractionated antivenom showed higher neutralizing ability against a coagulant effect than AS-fractionated antivenom. It has been observed, however, that polyvalent antivenom is relatively inefficient in the neutralization of edema-forming activity of Central American crotaline venoms.[3,6,8,9] It was postulated that, owing to the presence of non-immunoglobulin proteins in antivenom fractionated with AS, incubation of venom and antivenom results in the proteolytic release of pharmacologically active peptides from plasma protein precursors, thereby making difficult the neutralization of edema.[9] Recently, the Egyptian antitoxin antibody producer has been change the production protocol from ammonium sulfate into caprylic acid. We will not take on the product potency in our consideration because the major problem of this industry is focused on the side effect which arose in patients in response to the animal protein, mainly the impurities of non-gammaglobulin and/or antibody aggregates. So, the main aim of our work is the comparison between Egyptian horse

antitoxin F(ab)$_2$ antibody fractionated by AS and CA purification protocols. The comparison will include product purity, anticomplementary activity, and endotoxin content.

EXPERIMENTAL

All of our antitoxin F(ab)$_2$ antibodies were purchased from (Vacsera, Cairo, Egypt), anti-tetanus, anti-diphtheria, antivenom products (expired by 2000 or produced in 2005).

Protein Content

Total protein concentration and albumin content were determined as previously reported.[6]

Turbidity and Protein Aggregates

The turbidity of all undiluted antibody products was evaluated by recording the absorbance at 590 nm using a Shimadzu UV-160 spectrophotometer.[6] To detect the protein aggregates, a sample (500 μL) from each antibody product was applied to a Sephadex G-200 column (1.5 x 50 cm) pre-equilibrated with 50 mM sodium phosphate/150 mM sodium chloride/0.02% sodium azide at pH 7.2. The flow rate was maintained at 3 mL/tube. To estimate the molecular weight, the following protein molecular weight standards were used: blue dextran (2,000,000), human IgG (150,000), and human serum albumin (68,000). The elution profiles were determined by recording the absorbances at 280 nm. The fractions were stored at 4°C for protein screening, SDS-PAGE, and ELISA analysis.

The percentage of undigested horse IgG was evaluated by using Protein G (Amersham Bioscience). Each antibody product was dialysed overnight against 100 mM Tris.HCl pH 8.5/150 mM NaCl; the dialyzed product was concentrated to the original volume. Then, the immunoprecipitation was preceded according to the manufacturer's instruction.

SDS-PAGE

A non-reducing 12% SDS-PAGE was performed as described.[10]

Enzyme Linked ImmunoSorbent Assay (ELISA)

ELISA plates (Costar) were coated with antibody products or the fraction from gel chromatography at 100 ng/50 μL/well. Plates were incubated at

room temperature for 3 h, then at 4°C overnight. Primary and secondary antibodies were used at dilutions of 1:1,500 (goat anti-horse IgG, labeled with biotin, KPL, USA) and 1:3,000 (Streptavidin-HRP, Bohringer-Menhim, Germany), respectively. TMB 3,3′,5,5′tetramethylbenzidine substrate was used to develop the peroxidase and the color change was recorded at 490 nm. Each reading represents a mean of 3 absorbances ± standard deviation.

In Vitro Anti-Complementary Assay

In vitro anti complementary assay (ACA) was assessed according to Refs. [3,6]. In brief, 2 mL of various additions of antivenoms were added to 0.5 mL of fresh human serum previously diluted 1:20 with barbital buffer, pH 7.6. After 1 h of incubation at 37°C, 100 μL of sheep erythrocytes, previously sensitized with rabbit anti-sheep erythrocyte antibodies, were added, and the mixture was incubated 1 h at 37°C. Then, 2 mL of barbital buffer was added, the samples were centrifuged, and the absorbances of the supernatants were recorded at 540 nm. The ACA activity was expressed as the inverse of the dilution that reduced complement activity by 50%, taking as 100% the complement activity of serum samples that were incubated with barbital buffer instead of antivenom.

Endotoxin Content

Lipopolysaccharide (LPS) has been evaluated by using dot-ELISA.[11] In brief, one μL (10 μg/mL) of non-capsulated *Nisseria meningitides* strain M986-NCV-1 LPS (a kind gift of Dr. C. M. Tsai, FDA, Bethesda, Maryland) was loaded onto a nitrocellulose sheet in parallel with digested antitoxin antibody (the antibody products were added to reducing sample buffer, and boiled for 10 minutes at 100°C. An equal volume of Proteinase K (2.5 mg/mL) was added to the precooled antibody samples, incubated for 60 min at 60°C). The sheet was immediately dried, then blocked with 3% gelatine-0.1% Tween 20. Diluted IVIGs (VIGAM-S BPL, Herts, UK), (20 μg/mL) was added and the blot was incubated at room temperature, with shaking, for one h. After washing (1% gelatine-0.05% Tween 20), 1:2,000 goat anti-human IgG (H + L) $F(ab)_2$ alkaline phosphatase (PIERCE, Illinois, USA) was added and the blot incubated at room temperature for one hour. The blot was vigorously washed, then developed with Sigma FAST substrate 5-bromo-4-chloro-3-indolylphosphate *p*-Toluidine salt/nitro blue tetrazolium chloride, (BCIP/NBT) for 15–20 minutes.

RESULTS AND DISCUSSION

Currently, the major problem facing therapeutic antibodies is the side effects. We have no clear understanding of from where they arise. Is it from the antibody purification protocols used, the kind of host animals used for production, is it from whole IgGs over its fragments, or is it from the techniques used for side effect assessment and evaluation?

The chromatographic profiles of both product kinds of horse antitoxin antibodies, when subjected to Sephadex G-200, are shown in Figures 1 and 2. Both products contain a large peak corresponding to protein with short elution volumes, which may represent protein aggregates. In addition, the product produced by AS showed peaks which were missed in CA fractionated products, corresponding to protein of molecular weights 70, 45, and 25kD, probably albumin, and many other serum protein fragments, because they did not have any ELISA (Fig. 1) or western blot (data not shown) activity. Electrophoretic and chromatographic profiles and ELISA showed that the most fractioned protein of the caprylic acid product represents the horse antibody or its fragments (Figs. 3 and 4). The ammonium sulfate fractionated products had less intact IgG, compared to caprylic acid fractionated products (Table 1). Non-reducing electrophoresis comparison of these two products also showed conspicuous differences (Figs. 3 and 4) which were consistent with the Sephadex G-200 elution patterns (Figs. 1 and 2). The levels of

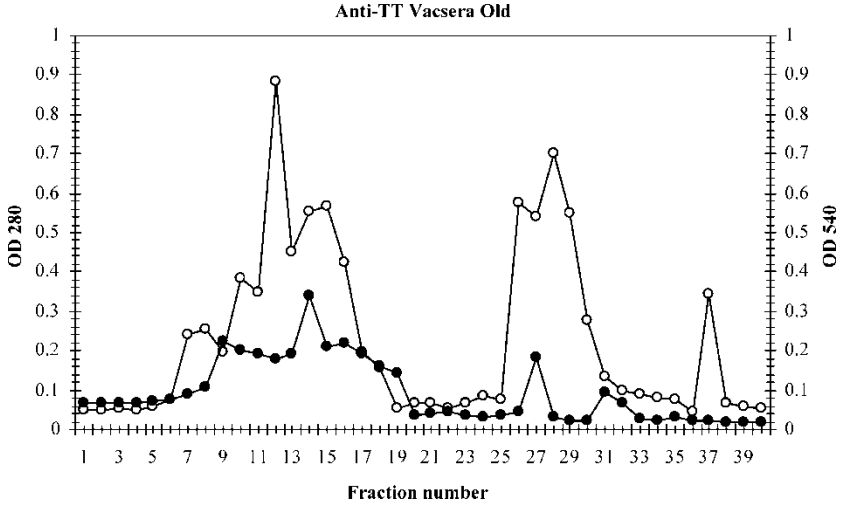

Figure 1. Typical elution profile of the representative antivenom antibody preparations following chromatography on Sephadex G-200 column at 3 mL/tube in equilibration buffer (50 mM PBS/150 mM NaCl/0.02 sodium azide, indicates the aggregation, whole IgG, F(ab)$_2$ and its fragments, albumin, and other gamma-globulin proteins. Open and solid circles represent the protein and ELISA optical density, respectively.

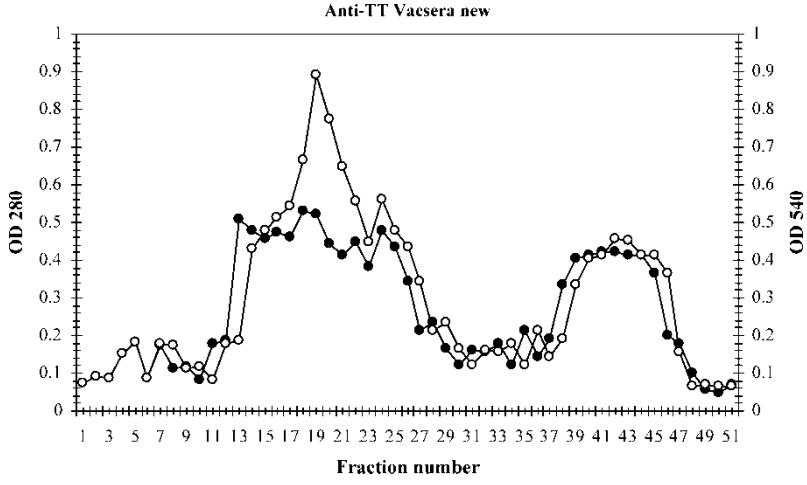

Figure 2. Typical elution profile of the representative antivenom antibody preparation fractionated by acrylic acid, following chromatography on Sephadex G-200 column indicates the aggregation, whole IgG, $F(ab)_2$ and its fragments. Open and solid circles represent the protein and ELISA optical density, respectively.

gamma globulins were similar, but the product fractionated by AS had a higher amount of non-gamma globulin fragments. These contaminating fragments were greatly reduced in the CA fractionated products.

The CA fractionated products contained significantly fewer aggregates and turbidity in comparison with AS fractionated products (Table 1). These results were nearly identical to the anticomplementary data of both products, and might suggest why AS fractionated products induce more ACA than CA fractionated products. The endotoxin (ET) results show that both kinds of products contain detectable endotoxin contents. The new and old anti-tetanus antibody products contained endotoxin (Fig. 5), while the other products did not. However, anti-gas gangrene product (imported from India) has a pyrogenic level of endotoxin.

ET (pyrogens) is known to cause fever in humans and other mammals at very low concentrations and irreversible septic shock at higher doses. The threshold level of ET for intravenous applications is set to 5 endotoxin units (EU) per kg of body weight per hour by all pharmacopoeias.[12] The term EU describes the biological activity of an ET; 1 EU usually corresponds to 100 pg ET. Therefore, it is essential to reduce the ET level in solutions used for intravenous administration to concentrations tolerable to the organism.[13] Recently, a WHO workshop on animal therapeutics antibodies placed the endotoxin and virus contamination in the same category of product safety,[5] while Silva de Freitas[14] recommended a new methodology to reduce and/or remove the pyrogenic factor from antitoxin $F(ab)_2$ products.

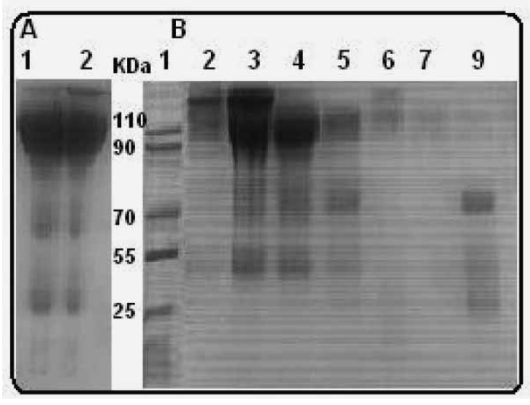

Figure 3. 12% of non-reducing SDS-PAGE analysis of ammonium sulfate fractionated F(ab)$_2$ products (anti-tetanus, anti-diphtheria, antivenom). Lanes 1 and 2 in part A represent the whole of the anti-tetanus and antivenom antibody products, while lanes 2-9 in part B represent the antivenom fractionated product. We have chosen only the antivenom results for representation, but the two other products were completely the same. Lane 1 in B indicates reducing protein standard of Pharmacia. The gel was stained with Coomassie blue.

Generally, our results are in agreement with the previous results.[3,6] They indicate that the CA fractionation is superior to AS fractionation with respect to product purity, potency, and yielded antitoxin with less anticomplementary content. The anti-complementary agent in the products may refer to the

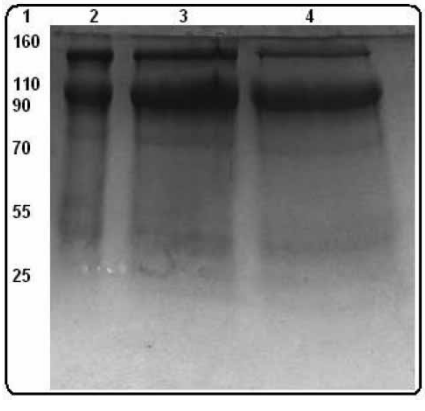

Figure 4. 12% of non-reducing SDS-PAGE analysis of caprylic acid fractionated F(ab)$_2$ products (anti-tetanus, anti-diphtheria, antivenom). Lane 1 represents the protein standard, while lanes 2-4 represent anti-tetanus, anti-diphtheria, and antivenom products, respectively. The gel was stained with Coomassie blue.

Table 1. Properties comparison of antitoxin horse antibody products prepared by ammonium sulfate and caprylic acid protocols

Antivenom (AS, CA)	Protein (g/dL)	Albumin (g/dL)	Aggregates (%)[a]	Turbidity (A_{600})	wIgG (%)[b]	ACA (A_{540})
AS1	$4.75 \pm 0.13^{*}$	$0.64 \pm 0.03^{**}$	4.50	$0.046 \pm 0.002^{**}$	6.1	41 ± 8
AS2	4.73 ± 0.15	0.66 ± 0.02	5.45	0.043 ± 0.002	6.3	43 ± 8
AS3	4.71 ± 0.12	0.63 ± 0.01	4.73	0.033 ± 0.003	6.2	40 ± 7
CA1	$3.54 \pm 0.12^{*}$	$0.18 \pm 0.02^{**}$	2.78	$0.021 \pm 0.001^{**}$	8.1	32 ± 6
CA2	3.52 ± 0.11	0.16 ± 0.01	2.45	0.022 ± 0.002	8.3	35 ± 7
CA3	3.55 ± 0.11	0.15 ± 0.01	2.63	0.021 ± 0.001	7.8	32 ± 7

*, **Results were significantly different ($p < 0.05$, $p < 0.01$).

AS, CA ammonium sulphate and caprylic acid respectively.

[a]percentage of anti-serum protein corresponding to high molecular weight aggregates or

[b]to 150-160 KDA, as judged by column chromatography.

AS1, AS2, AS3 corresponding to anti-tetanus, anti-diphtheria, antivenom anti-serum $F(ab)_2$ products produced (expiry date was 2000) by ammonium sulfate, respectively. CA1, CA2, and CA3 same names but produced by caprylic acid (production date 2005).

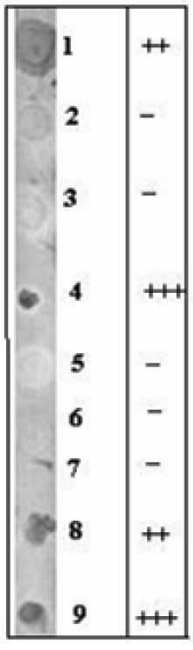

Figure 5. Dot-ELISA for detection the endotoxin in antitoxin antibodies products (1-antitetanus*, 2-anti-diphtheria*, 3-anti-snake venom*, 4-antitetanus**, 5-anti-diphtheria**, 6- anti scorpion**, 7-antisnake venom**, 8-anti-gas gangrene**, 9-Meng C LPS, 10pg/uL). * Before year 2000 products, ** year 2005 products, - LPS not detected, ++ less than 10 ng, +++ 10 ng.

product aggregates, non-gamma-globulin protein, and/or turbidity. Various workers have described the ACA test as a predictor of ACA *in vitro*,[1,3,8,15,16] although recently, Leon has raised doubts on the clinical significance of the *in vitro* ACA test as a predictor of ACA *in vivo*[17]

In conclusion, by using gel-filtration, ELISA, SDS-PAGE, and *in vitro* anticomplementary assays to make comparisons between AS and CA fractionated antitoxin quality, suggest that the replacement of AS with CA yields a significant improvement in most products′ biochemical characteristics. The digestion and incubation times may need to be redetermined to reduce the total intact IgG content. In addition, precautions should be taken to reduce the pyrogen content.

ACKNOWLEDGMENTS

I wish to thank Mr. H. Abd Elkader and W. Wazier for technical assistance. This study was partially funded by grants from the (WHO/EMRO/

COMSTECH-04/13, and Egypt/USA JF No.65 for E.M.R.). The author thanks Dr. M. Kelker for language corrections and for his suggestions.

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Received April 7, 2006
Accepted May 14, 2006
Manuscript 3200