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Evaluation of Albumin Content in Hyperimmune Plasma and Antivenom for Assessment of Purity

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ABSTRACT

One of the important issues for quality control of biological products is purity. In most cases, electrophoresis is used to determine the purity of the proteins and the quantity of related band is determined by densitometry and respective software. In order to check the purity of hyperimmune plasma and antivenom bulks, we have evaluated the albumin content of these products. Sixty samples were divided into two groups: the hyperimmune plasma group that containing thirty bulks of monovalent and polyvalent anti-snake, polyvalent anti-scorpion and anti-diphtheria toxin plasma, and plasma-derived antivenoms group, comprising thirty bulks sera obtained from the purification of relevant plasma bulks. The albumin purification from horse normal serum was performed through heat shock strategy and used as reference for the quantification of albumin content. After electrophoresis of samples, the gel images were analyzed using Image J software and the amount of protein in relevant band was determined by calculating the curve area and comparison with reference albumin. The results revealed significant changes on albumin level in hyperimmune plasma samples. Two out of 30 plasma samples had lower and sixteen samples had a higher albumin than normal levels. Using the Image J, we could not detect any residue of albumin in antivenom sera. The results demonstrated effectiveness of purification protocol for removal of albumin during antivenom production process. However, development of a quantitative evaluation method along with the semi-quantitative method used in this study can help the accuracy of these results.

Keywords: Albumin, Antivenom, Image J, Electrophoresis

INTRODUCTION

The only approved method for the treatment of venomous animal bites and some infectious diseases is the use of heterologous antibodies [1]. These products are most often prepared from hyperimmune horse plasma by different refinement strategies. Manufacturing process design should be in a way that refines the immunoglobulins from residual non-immunoglobulins in just a few simple, effective, and easy steps in order to provide a product with acceptable physicochemical properties and protective activity [2].

The purity of antivenom is ordinarily assessed based on the physicochemical properties of the antivenom which demonstrate the quantity of active ingredients, *i.e.* immunoglobulins, and the risk of hypersensitivity that impurities such as albumin can cause [3,4]. The physicochemical purity of antivenoms can be improved by careful purification methods, especially immunoglobulins (or their fragments) fractionation and albumin removal, which emphasized in all related guidelines [5-7].

Albumin is the main protein of mammalian blood plasma. It is relatively large (molecular mass of around 66kDa) and carries a net negative charge. Albumin is a versatile protein with exceptional ligand-binding capacity, making it a carrier molecule for many metabolites, metals, nutrients, drugs, and other molecules [8]. Horse albumin is a recognized causal agent of hypersensitivity to equine antivenom, resulted shortness of breath and anaphylactoid reaction in patients [1, 9]. The finding, therefore, points to the need for more stringent quality control of the purity of antivenoms, with albumin content ideally not exceeding 1% of total proteins, in line with the WHO guideline for antivenom production [1]. However, the protein content and antivenom purity are expected to vary between antivenom batches, and this limit may be subject to rigorous consistency checks from time to time [10,11].

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Normally, the therapeutic sera quality control laboratories check the purity of the obtained product using the SDS polyacrylamide gel electrophoresis (SDS-PAGE) technique. For this purpose, the relevant bands of IgG, $F(ab)_2$, Fab, and plasma protein contaminants (especially albumin) are checked [1]. In general, non-reducing SDS polyacrylamide gel electrophoresis generates qualitative information about the presence of intact immunoglobulins, the amount of digestion products, soluble protein aggregates, and contaminant albumins [1,10]. SDS-PAGE under reducing conditions is commonly used for assessing the amount of immunoglobulins and their derivatives through the direct insight of immunoglobulin heavy chains intact or digested [1].

Based on this information, the aim of this study was to evaluate the content of residual albumin in various antivenom bulks before the formulation process. Here, we investigated the image analysis method that allows the quantification of albumin in the bands of electrophoresis gels using the purified horse albumin as protein standard.

MATERIALS AND METHODS

Normal and Hyperimmunized Horse Plasma and Antivenom

Normal horse plasma was provided by department of venomous animals and antivenom production of Razi Vaccine and Serum Research Institute and applied for albumin purification.

Four types of antivenoms were used in this study: (a) polyvalent anti-snake venom serum (n = 21). These antivenoms were obtained from the plasma of horses hyperimmunized against five venoms of Iran origin: *Vipera lebetina*, *Vipera albicornuta*, *Echis carinatus*, Pseudocerastes persicus, and *Agkistroden halys*; (b) polyvalent anti-scorpion venom serum (n = 7), from the plasmas of horses hyperimmunized against the venoms of *Andoroctonus crassicauda*, *Buthotus saulcyi*, *Buthotus schach*, *Odontobothus doriae*, *Mesobuthus eupeus* and *Mesobuthus*; (c) monovalent anti-snake venom serum (n = 1) and (D) *anti-diphtheria* toxin *serum* (n = 1), obtained from the plasmas of horses hyperimmunized against the venom of *Naja Naja oxiana* and diphtheria toxoid respectively. Every single one of the antivenom types constituted by $F(ab)_2$ fragments was fractionated by ammonium sulfate precipitation. All these plasmas and antivenoms were manufactured by Razi Institute, and were used in albumin measurement for comparison purpose in this study.

Horse Albumin Purification

The normal horse plasma was diluted 1:1 with water and precipitated by the addition of caprylic acid (0.04 M) for initial preparation of albumin. The caprylate was added slowly but steadily with constant stirring at room temperature and allowed it stir for an additional 15 min. The mixture was divided into two parts, the first was heated at 70 °C for 60, the second at 75 °C for 10 min and their pH was measured and adjusted with 1 N HCl to give the pH value of 4.2. The resulting mixtures were kept for overnight at 4 °C and then centrifuged at 2500 rpm for 10 min at 25 °C to separate precipitate consisting *of* globulins. The supernatants containing mainly albumin were stored at -30 °C until used.

To further albumin purification, the selected supernatant was subjected to 25% saturated ammonium sulfate (13.6 g dL^{-1}). The powder was gradually added and thoroughly mixed under gently magnetic stirring until precipitate formation was achieved. The supernatant was obtained by centrifugation at 2500 rpm for 10 min, dialyzed overnight at 4 °C against water.

Protein Determination

The protein concentration of purified horse albumin was measured according to the Lowry *et al.* [12] method using bovine serum albumin as standard.

Characterization of Purified Horse Albumin

The purity of the albumin preparation, obtained by the relevant purification procedures, was analyzed under non-reducing SDS-PAGE mini gels of 10% polyacrylamide, as adapted from Laemmli [13]. Bovine serum albumin (BSA, 1 & 5 mg ml^{-1}) was used to compare *protein* purification efficacy and molecular weight determination. The Gels were stained with Coomassie Brilliant Blue R-250.

Detection and Quantification of Albumin in Hyperimmune Horse Plasma and Antivenom Batches

To evaluate of residual albumin concentration in antivenom samples, protein electrophoresis is used as a standard method for antivenom purity assessment combined

with the determination of total albumin amount of hyperimmunized plasma samples related to each antivenom. Plasma and antivenom samples was separated by 12.5% SDS-PAGE under non-reducing conditions. Protein bands were visualized as a mentioned above. To quantify albumin content in each sample, all SDS-PAGE gels were imaged and then loaded into Image J. The "Adjust Brightness/Contrast" option was used to emphasize bands after images were converted to 32-bit resolution. A box was drawn over the first control (purified albumin) and selected as the first lane using "Analyze - > Gels - > Select first lane" in order to quantify the intensity of the bands. The remaining lanes were used to select sequential lanes by placing boxes on them and selecting the "Analyze - > Gels - > Select next lane" option. The Plot lanes function was used to plot the intensity of each band. The straight line tool was selected from tool menu and the area under the peak was marked off for the first lane and this process repeated for the next lanes and then using "wand" function was selected each band peak; this outline each of the peaks in yellow. After selecting the last peak, was opened another window labeled results. This window contains all of the values for the areas under each band peak selected from the plots. Then results were transferred into Excel spreadsheet to use for further calculations.

The peak intensity of purified albumin was used as reference to quantify albumin protein in hyperimmune plasma and antivenom samples. The amount of albumin protein band (P, μg) in each lane of sample was calculated from the multiply of albumin band AUC (Area Under the Curve) in concentration (C, μg/10 μl) of purified albumin divided by purified albumin AUC (Eq. (1)).

$$P = AUC_{AS}*C/AUC_{PA}$$

AUC_{AS}: Area under the Curve of albumin sample

AUC_{PA}: Area under the Curve of purified albumin

RESULTS

SDS-PAGE Analysis of Purified Horse Plasma Albumin

The results obtained electrophoretic analysis of extracted caprylate supernatants on 10% polyacrylamide gel showed that changes in temperature and time had almost no effect on the amount of extracted albumin. However, some of non-albumin undesirable proteins and one broad predominant band of nearly 66 kDa horse albumin have been detected (data not shown). To improve the purification of horse albumin, ammonium sulfate precipitation was followed on obtained supernatant over a shorter period of time. The comparative results suggested that the profile and band intensity of horse albumin and BSA 98% (5 mg ml^{-1}) agree well. However, the amount of unwanted proteins and albumin band intensity are respectively more negligible and slightly higher than that of BSA (Fig. 1).

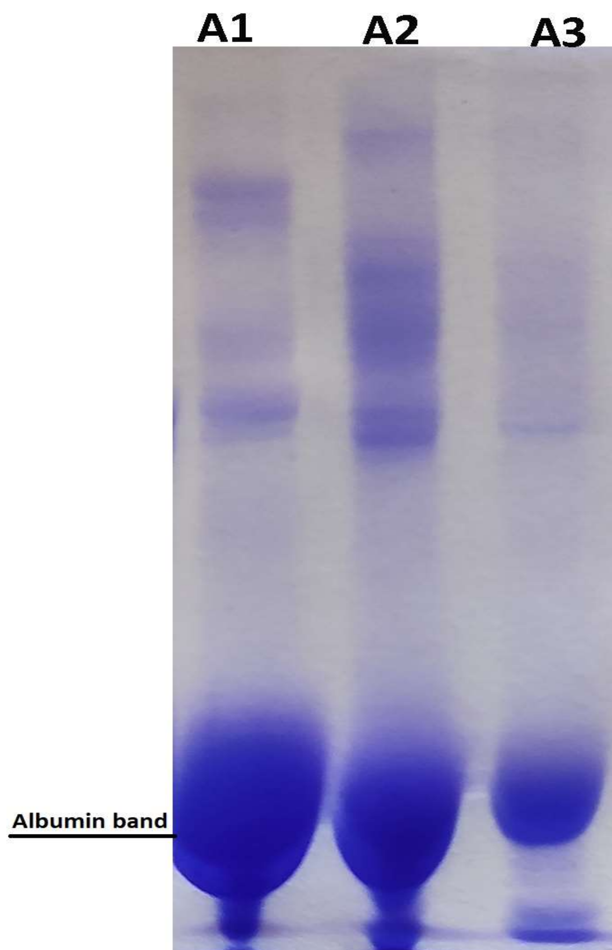

Fig. 1. SDS-PAGE of ammonium sulfate prepared albumin from heat shock supernatant. Aliquots of albumin preparations were separated by electrophoresis in 10% acrylamide gels under non-reducing conditions. Proteins were stained with Coomassie brilliant blue R-250. A1: Horse serum albumin, A2: BSA (5 mg ml^{-1}), and A3: BSA (1 mg ml^{-1}).

Quantification of Horse Albumin Protein in both Hyperimmune Plasma and Antivenom Samples

The unknown concentration of albumin protein in hyperimmune plasma and antivenom samples were obtained from 8 SDS-PAGE gels stained with Coomassie Brilliant Blue and analyzed by Image J (Fig. 2 and supplementary materials Fig. 1). The purified albumin was electrophoresed along with the others, and albumin band in all samples quantified by densitometry. The protein peak area in the densitometry profile was correlated with the protein load and used as a signal in the most analysis.

The albumin protein in the bands of the gel ranged from 0.24 to 0.56 μg per band for hyperimmune plasma samples corresponding to concentration from 2.4 to 5.6 g dL^{-1} of plasma by applying the dilution factor. However, albumin bands could not be detected and calculated in any of the antivenom sera (Table 1).

DISCUSSION

The fundamental principle for the all biological products manufacturing, such as antivenoms, is the compliance to GMP and other international standards [14].

Poor GMP, particularly during the plasma fractionation and subsequent manufacturing processes, frequently results in hypersensitivity reactions, pyrogenic reactions, and other adverse events because protein aggregates, endotoxins, and other impurities may be present [15]. The presence of protein impurities such as albumin in antivenom serum is probably due to inadequate purification system to separate $F(ab)_2$ from other plasma proteins at the early stage of antivenom manufacturing process [10]. Equine serum albumin is one of the most concerning aspects of hypersensitivity to horse serum-based antivenom, which may go into shock and other respiratory symptoms [1,9].

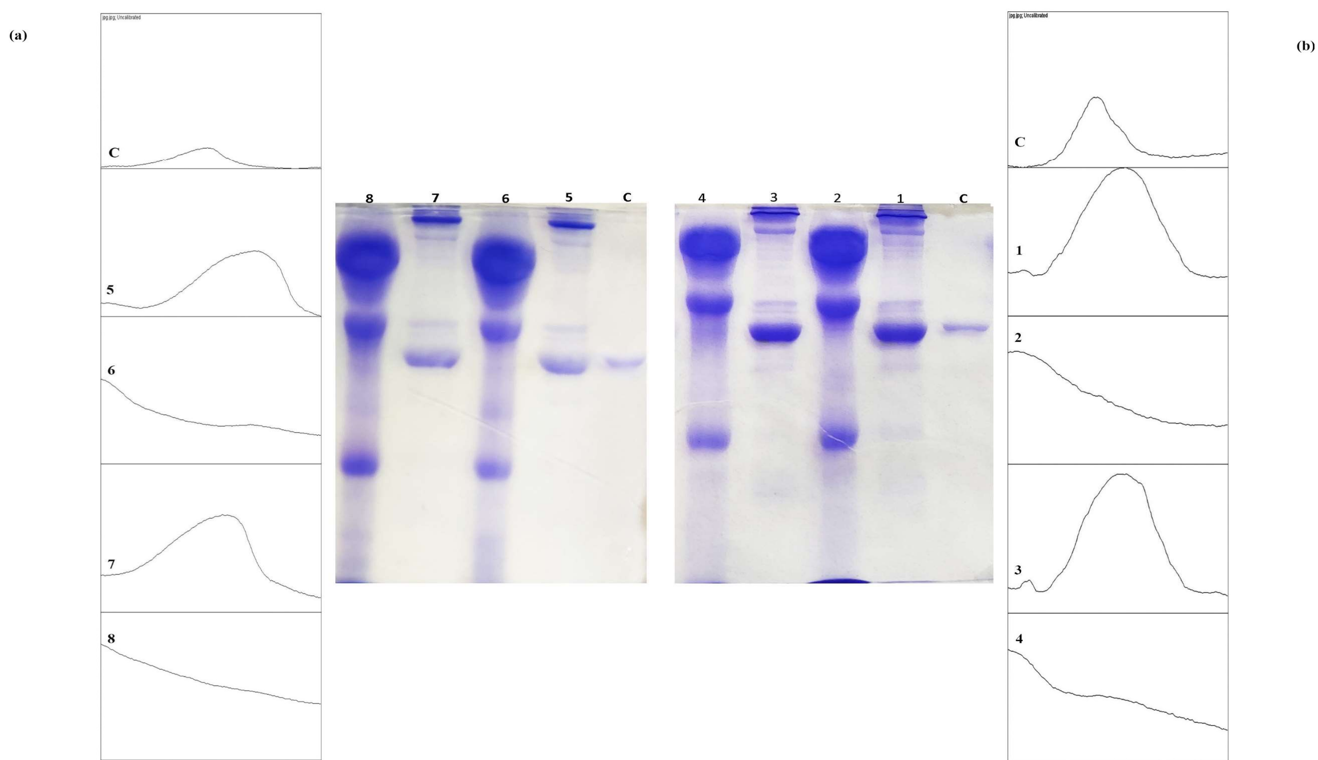

Fig. 2. SDS-PAGE of hyperimmune plasma and antivenom serum samples in order to albumin content band quantification through Image J (densitometry). Horse purified albumin (C) used as protein reference. Out of a total of 60 samples, only eight are given as examples. anti- diphtheria *toxin* hyperimmune plasma (1), anti-diphtheria toxin serum (2), polyvalent anti-snake hyperimmune plasma (3), polyvalent anti-snake venom serum (4*)*, monovalent anti-snake hyperimmune plasma (5), monovalent anti-snake venom serum (6), polyvalent anti-scorpion hyperimmune plasma (7), polyvalent anti-scorpion venom serum. (a) and (b) are the graphs of the area under the curve corresponding to the SDS-PAGE figures from Image J software.

Table 1. Quantification of Albumin in Hyperimmune Plasma and Antivenom Serum Samples Based on SDS-PAGE Analysis by Image J

Protein content of albumin bands (μg)	AUC	Type of sample	Sample
0.6	13619	Purified albumin	Control
2.4	54220	Anti-diphtheria	Plasma
-	-		Antivenom
2.6	58281	Anti-snake polyvalent	Plasma
	-		Antivenom
2.6	58413	Anti-snake polyvalent	Plasma
-	-		Antivenom
2.6	59987	Anti-snake polyvalent	Plasma
-	-		Antivenom
0.6	4314	Purified albumin	Control
4.2	30122	Anti-snake polyvalent	Plasma
-	-		Antivenom
4	29051	Anti-snake polyvalent	Plasma
-	-		Antivenom
4.4	31774	Anti-snake polyvalent	Plasma
-	-		Antivenom
4.1	29699	Anti-snake polyvalent	Plasma
-	-		Antivenom
0.6	7827	Purified albumin	Control
2.6	33920	Anti-snake polyvalent	Plasma
-	-		Antivenom
3.5	45987	Anti-snake polyvalent	Plasma
-	-		Antivenom
3.4	44164	Anti-snake polyvalent	Plasma
-	-		Antivenom
3.7	48845	Anti-snake polyvalent	Plasma
-	-		Antivenom
0.6	8348	Purified albumin	Control
3.6	49697	Anti-snake polyvalent	Plasma
-	-		Antivenom
2.4	33686	Anti-snake polyvalent	Plasma
-	-		Antivenom
2.8	39579	Anti-snake polyvalent	Plasma
-	-		Antivenom
0.6	5133	Purified albumin	Control
4.4	37616	Anti-snake polyvalent	Plasma
-	-		Antivenom

Table 1. Continued

4.4	37311	Anti-snake polyvalent	Plasma
-	-		Antivenom
5.6	48182	Anti-snake polyvalent	Plasma
-	-		Antivenom
5	42772	Anti-snake polyvalent	Plasma
-	-		Antivenom
0.6	5372	Purified albumin	Control
3.2	28519	Anti-snake polyvalent	Plasma
-	-		Antivenom
3.4	30346	Anti-snake polyvalent	Plasma
-	-		Antivenom
3.2	29056	Anti-snake polyvalent	Plasma
-	-		Antivenom
0.6	3478	Purified albumin	Control
4.6	26729	Anti-snake monovalent	Plasma
-	-		Antivenom
4.8	27629	Anti-scorpion polyvalent	Plasma
-	-		Antivenom
4.5	26123	Anti-scorpion polyvalent	Plasma
-	-		Antivenom
4.4	25342	Anti-scorpion polyvalent	Plasma
-	-		Antivenom
0.6	5133	Purified albumin	Control
4.4	37616	Anti-scorpion polyvalent	Plasma
-	-		Antivenom
4.4	37311	Anti-scorpion polyvalent	Plasma
-	-		Antivenom
5.6	48182	Anti-scorpion polyvalent	Plasma
-	-		Antivenom
5	42772	Anti-scorpion polyvalent	Plasma
-			Antivenom

To analysis of albumin content in the hyperimmune plasma and antivenom serum samples, the albumin was purified from normal horse serum. There are different methods for albumin purification, including plasma fractionation with ethyl alcohol [16-18], the combination of the Cohn method with liquid chromatography [19], affinity precipitation [20], heat shock method [18], TCA/Acetone precipitation method [21], ion exchange chromatography [22,23], *etc*. In this study, albumin purification was performed under slight modification of Cohn and Chanutin heat shock procedure [24]. On comparing the other plasma proteins, albumin is stable against denaturation at high temperatures by a little amount of long-chain fatty acids such as caprylic acid. In addition to acting as an albumin stabilizer, caprylic acid can also function *as* a precipitating agent of denatured globulins. This may be because the protein has an

interaction with caprylic acid that is insoluble at pH 4.2 [24]. It is important to note that initial precipitation using the heat shock method was extremely effective in albumin purification and removing major impurities. A clear conclusion for obtaining purified albumin is that it is inadvisable to apply ammonium sulfate for the next step of purification because of its negligible effect in removing of unwanted high molecular weight proteins.

The purity assessment is one of the standard used to guarantee the quality of biological products. In most cases, physicochemical techniques such as chromatography and electrophoresis are used as rapid and acceptable methods to determine purity [25].

In this study, we investigated the quantity of residual albumin in the antivenom serum bulks using SDS-PAGE analysis under non-reducing condition as an in process quality control test and simultaneously compare it with albumin amount in electrophoretic profile of related hyperimmune plasma bulks. Electrophoresis gels were analyzed through Image J software for the quantification of albumin band protein in plasma hyperimmune and antivenom serum samples using the band of purified albumin as a reference. The peak area of the protein in the densitometry profile was correlated with the protein load. In most densitometry analyses, the signal used is the peak area [26-28]. Because the obtained correlations frequently result in inaccurate estimations of protein load, the volume of the peak is rarely utilized [29]. Using the information of protein load per albumin band and the equal load of samples in the SDS-PAGE gel (10 μl), the protein amount per band was correlated with the peak area using Microsoft Excel 2016. The reference range of horse serum albumin concentration was dictated as 2.6-3.7 g dL^{-1} [30]. Our results showed, regardless of the type of hyperimmune plasma, almost significant changes in the albumin concentration of a number of plasma batches, dragging it to outside the normal range. Of 30 plasma samples checked for albumin content, 12 (40%) were in normal range and 2 (6.6%), 16 (53.3%) below and above the normal limit, respectively. Previously, a decrease in serum albumin levels was reported [31]. This is consistent with the profile of albumin, which is regarded as a negative acute phase protein due to the fact that albumin serum concentration decreases during the inflammatory response [32-34].

Also, there may be several explanations for the increase in albumin plasma, but we guess the main reason is the failure of red blood cells to return to the blood circulation of horses after bleeding stages. Perhaps the use of plasmapheresis technique can help solve this problem. It should be noted that the albumin band could not be identified in any of the antivenom samples with any level of primary albumin in plasma batches, and this can promise the efficacy of the purification method for the preparation of albumin free antivenom. The design of our study was insufficient to determine which protocol would be most effective, despite the fact that these findings have a great deal of potential for use in the future to promote animal welfare in horses kept in programs that produce antivenom. It can be suggested to use quantitative commercial kit simultaneous to electrophoresis method which can give us a measurement of accuracy.

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