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Comparison of Different Albumin Removal Methods for Evaluation of Human Serum Proteome

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Abstract

***Background*:** Proteomics examines the protein content of a cell, tissue or physiological fluid in certain developmental or pathological conditions. Serum, a rich source of protein, is easily accessible and represents the physiological and clinical status of individuals. Emergence of a disease can cause qualitative and quantitative changes in the concentration of certain proteins in the serum. Nevertheless, separation and examination of low abundance proteins at presence of high abundance proteins such as albumin and immunoglobulin, which comprise over 60% of total protein, are limited.

***Materials and Methods*:** In this experimental study, in order to remove albumin, serum samples from four different removal methods including two trichloroacetic acid (TCA) precipitation, ammonium sulfate fractionation and using specific kits to remove albumin and immunoglobulin were compared.

***Results*:** The results indicated that precipitation and fractionation techniques considerably remove albumin. Due to its specific performance, using kit removes larger amounts of albumin and immunoglobulin. However, the protein patterns derived from different albumin removal methods were different.

***Conclusion*:** It is suggested that several albumin removal methods should be used during the examination of serum protein content.

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Introduction

Proteomics studies the structure and function of proteins, and changes after their translation and interactions with other molecules. One of the main goals of proteomics is to discover biomarkers, the molecules that show the physiological changes of cells, tissue or organisms compared to the control sample and are objectives to diagnose the diseases and design drugs for treatment [1]. Body fluids such as serum are very important in predicting and treating a disease and changes in the expression of some proteins in it may be indicator of disease and proteomics technique provides a unique opportunity to analyze disease-related changes in the protein levels [2]. In serum, observation and identification of low concentration proteins are being disturbed by high concentration proteins such as albumin and immunoglobulin. One of the most common proteomics methods is based on two-dimensional electrophoresis technique. High concentration protein avoids optimal focusing (first dimension of electrophoresis) and limits the capacity of low abundance proteins. Therefore, removal of these proteins will lead to better identification of low concentration proteins [3]. Within the last decades, many efforts have been put to remove albumin as the most abundant serum protein. Most of these efforts are the methods based on high tendency of albumin for special colors like cibacron blue and its derivatives [4-6]. Another method is a matrix with tendency for polypeptide which is able to remove albumin with immunoglobulin [7]. In this method, it is likely that some proteins will be nonspecifically eliminated due to their binding to resin and the proteins which are attached to albumin and immunoglobulin. Using trichloroacetic acid is a general method for precipitation of proteins. In this method, the complex acid constitutes with serum albumin is soluble in organic solvents [8].

Debro and Korner have reported that the albumin which is precipitated with trichloroacetic acid is soluble in organic solvents [9]. Race has used 80% acetone as an organic solvent along with trichloroacetic acid to separate albumin from serum globulin [10]. Rodkey used ethanol and trichloroacetic acid simultaneously to separate globulins from albumins [11]. Presence of high concentration proteins in most biological fluids will reduce the capacity of using analytical techniques. In this study, different methods of removing albumin from serum were investigated for improvement and reproducibility of protein patterns.

Materials and Methods

In this experimental study, serum samples of patients with psoriasis were collected from laboratory and various albumin removal methods were examined and evaluated. Strips IPG, CHAPS, ampholyte, TEMED, DTT and urea were prepared from Bio-Rad Company and other

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materials and solvents were prepared from Merck Company of Germany.

Albumin Removal Methods

Ammonium Sulfate Fractionation method: Concentration of salt in which the proteins are precipitated varies from one protein to another. Therefore, different concentrations of salt can be used to separate proteins to different fractions. In this study, serum proteins were fractionated using saturation percentages of 30, 50, 70 and 90% of ammonium sulfate. Then, the protein content in each fraction was evaluated through two-dimensional electrophoresis technique, after desalinating with phenol and measuring protein concentration. For this purpose, 30 ml of serum reached a volume of 500 ml through phosphate buffer saline. Then, it reached saturation level of 30% after adding ammonium sulfate salt. After gentle vortexing, the samples were kept at room temperature for at least one hour and then, they were centrifuged for 30 minutes at 10000 g and temperature of 20°C.

The supernatant was used for further fractionation with saturation levels of 50, 70 and 90% of ammonium sulfate [12]. The four obtained precipitates were desalinated using saturation phenol to perform two-dimensional electrophoresis. That is, first, the precipitates were washed with 300 µl of cold acetone water and was dissolved in 2 ml of condenser buffer (0.5 M Tris-HCl, 30% w/v sucrose, 1% w/v SDS, 1% v/v 2-mercaptoethanol) and then, 2 volumes of the sample was mixed and centrifuged with a volume of buffer saturated phenol (buffered phenol, pH =8). Phenol content phase was mixed with 5 volumes of 0.1 M ammonium acetate in cold methanol, and after centrifugation, the precipitates were washed with methanol and were prepared for electrophoresis [13].

Trichloroacetic acid precipitation: Albumin-trichloroacetic acid complex is soluble in organic solvents such as acetone. One method to remove albumin from serum is to use acetone/TCA to precipitate proteins. In this method, the albumin in the sample remains in the supernatant phase binding to trichloroacetic acid and soluble in acetone, and other proteins will be precipitated. 4 volumes of solution of 10% trichloroacetic acid in acetone were added to 15 ml of the serum and it was kept at 20°C for at least 90 minutes and then it was centrifuged at 4°C.

The sediment after being washed with acetone, as well as the supernatant was used for electrophoresis. In the second removal method through trichloroacetic acid, 15 ml of serum reached the volume of 90 ml by phosphate buffered saline and then, 10 µl of 100% trichloroacetic acid was added to it and it was washed with cold acetone after being kept overnight at temperature of 4°C and centrifuge as well as precipitation [14].

Albumin removal using Kit: Aurum mini protein kit was purchased from Bio-Rad Company. This kit contains some columns which act based on the specific adsorption of albumin and immunoglobulin. After the preparation of column, 60 µl serum was injected into the column top and after several times performing vortex and centrifuge in accordance with instructions, the resulting sample was collected in tube and was used for electrophoresis. Bradford method was used in all methods to determine protein concentration.

SDS-PAGE Electrophoresis Techniques: First, a volume of the sample was dissolved in a volume of denaturant buffer (60 mM Trise HCl, 25% v/v glycerol, 2% w/v SDS, 14.4 mM 2-mercaptoethanol, 1% w/v bromophenol blue) and was boiled for 5 minutes at temperature of 100°C and was placed on polyacrylamide gel with concentration of 12%.

Bromophenol blue was used as migration control and electrophoresis was conducted in concentrator and separator gel, respectively with 60 and 90 V in a PROTEAN III Xi tank from Bio-Rad Company.

Two-dimensional Electrophoresis: Protein samples were dissolved in 180 µl of rehydration buffer contains 8 M urea, 4% w/v CHAPS, 20 mM DTT, 0.2% v/v ampholyte and 0.001% bromophenol blue, and they were centrifuged after one hour. Gel strips with a length of 7 cm with linear pH 3-10 (Bio-Rad, Hercules, CA, USA) were placed in rehydration buffer containing samples in the Protein IEF Cell Bio-Rad machine for 16 to 14 hours redehydration. After rehydration, focusing was performed on 50 volts for 15 minutes, from 50 to 150 volts for 30 minutes, from 150 to 250 volts for 15 minutes and then, voltage linearly reached 4000 for 2 hours and finally, focusing stopped on 12000 volts/h. Before performing the second dimension, gel strips were balanced in 6 M urea solution, 2.5% w/v SDS, 0.375M (pH=8.8) Tris-HCl, 30% v/v glycerol and 130 mM DTT for 15 minutes and in the same buffer but containing 130 mM iodoacetamide instead of DTT for 20 minutes. The second dimension was conducted for 10 min at 60 V and for 40 minutes at 200 volts on the gel 12%.

For the emergence of protein spots, 2 approaches of silver nitrate staining without using glutaraldehydes and staining with coomassie blue 250 G were used. After staining, gels were scanned using densitometry (Bio-Rad) GS-800 for analysis. Then, presence or absence and the value of each protein spot were analyzed through Melanie 6 software.

Results

Investigation of fractionation with ammonium sulfate: The quantitative study of fractions 50 and 70% through determination of protein concentration revealed that after desalination with phenol, the average 68.7% of the proteins in fractions 50 and 70% were recovered (Table 1).

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Table 1. Total protein concentration and percentage of recovered proteins in 50 and 70% saturated ammonium sulfate fractions after desalination with phenol

The Total protein	Protein concentration in 50%	Portions recovered in 50%	Protein concentration in 70%	Portions recovered in 70%
2175 µg	67800 µg	31.2%	817 µg	37.5%

It is seen that in this method large amounts of protein is also eliminated, due to two steps of precipitation and then desalination. The quantitative study of fractions was performed by comparing the protein pattern obtained from two-dimensional electrophoresis. After desalination, the proteins existing in precipitates from the fractions 30% and 90% were directly examined, but fractions 50% and 70% were examined after diluting eight times due to their high concentration of proteins (Fig.1).

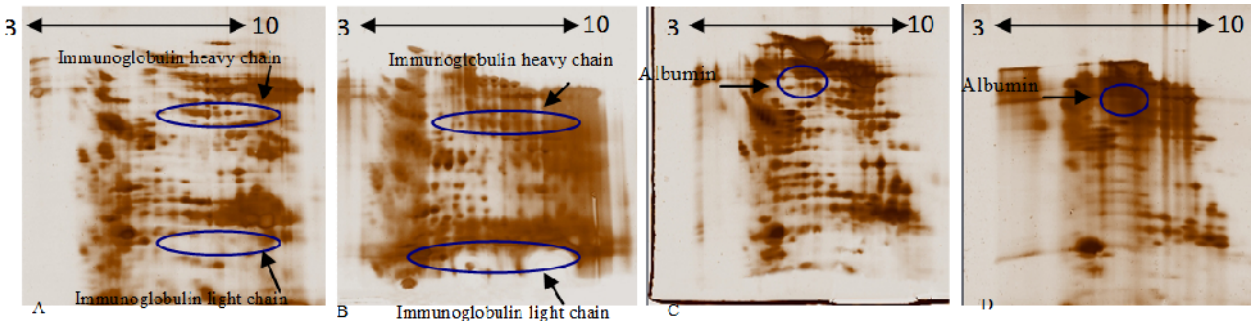

Figure 1. Comparison of two-dimensional protein patterns related to fractions 30 (A), 50 (B), 70 (C) and 90(D) percent of ammonium sulfate after desalination with phenol saturated. Proteins were separated on 7 cm IPG (pH 3–10) strips in the first dimension followed by 12% SDS-PAGE

Comparison of two-dimensional patterns of factions revealed the greatest albumin removal in fraction 70% of ammonium sulfate saturation (Fig. 1-C) and a small amount of it in fraction 90% of ammonium sulfate saturation (Fig. 1-D). In addition, 50% fraction of ammonium sulfate saturation contains heavy and light chain of immunoglobulin G without the presence of albumin and contains most other proteins (Fig. 1-B). The above results indicate that the maximum albumin and serum immunoglobulin will be eliminated respectively in fractions 50% and 70% of ammonium sulfate saturation. The quantitative results of concentration determination (Table 1) also confirm this. Examination of precipitation method using trichloroacetic acid/acetone: Sensitivity and specificity of this approach to remove albumin from serum was examined by one-dimensional electrophoresis (Fig. 2) and two-dimensional electrophoresis (Fig. 3) of the proteins in sediment and supernatant.

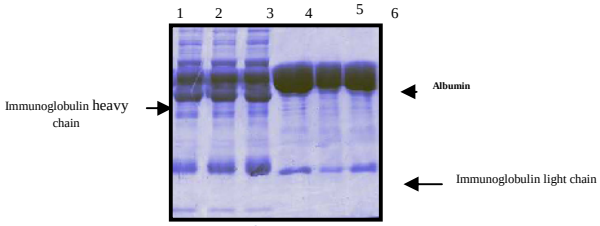

Figure 2. SDS-PAGE pattern of serum proteins precipitated with trichloroacetic acid/acetone. lines 1, 2 and 3 present the pattern of precipitated proteins and lines 4, 5 and 6 present the patterns of proteins in supernatant

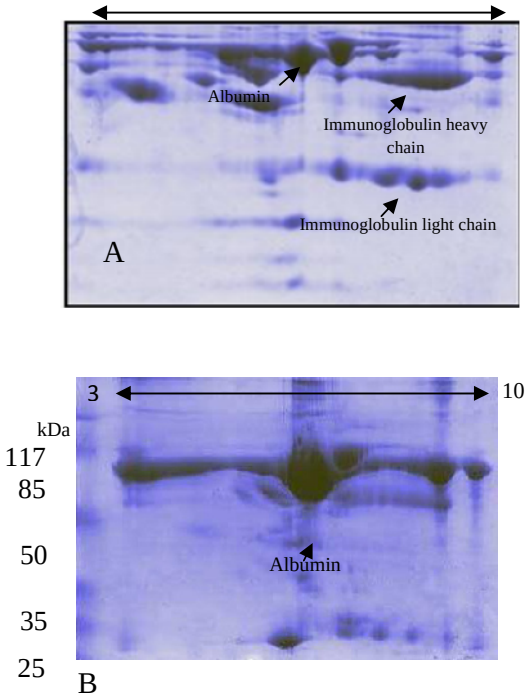

Figure 3. Two-dimensional pattern of serum protein precipitation with trichloroacetic acid / acetone. "A" represents the pattern of precipitated proteins and "B" represent the patterns of proteins in supernatant. Proteins were separated on 7 cm IPG (pH 3–10) strips in the first dimension followed by 12% SDS-PAGE

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Comparison of electrophoretic patterns of sediment and supernatant showed that using this method can separate significant amounts of albumin in the supernatant as TCA complex and soluble in acetone (columns 4, 5 and 6 in Figure 2 and pattern B in Figure 3) and their sediment includes other proteins existing in the serum (columns 1, 2 and 3 in Figure 2 and pattern A in Figure 3). Comparison of one-dimensional and two-dimensional patterns well shows the ability of this method to remove albumin. In addition, this method can be used for the isolation of globulins from albumins so that, one-dimensional and two-dimensional patterns would indicate the presence of immunoglobulin in the sediment (Figures 2 and 3).

One of the problems of using this method is low precipitated protein solubility and removal of some amounts of protein due to washing with acetone. This method is fast, easy and useable for high volume of serum. In the third method, 100% trichloroacetic acid with no acetone was used. The resulting precipitate got first dissolved in water and then, was washed with acetone to remove the remaining acid. In this method, a large amount of albumin was removed and the resulting two-dimensional gels show high ability to remove albumin (Fig. 4-B). Because of using water, larger amounts of other proteins are removed after the precipitation, which can be clearly observed when compared to two-dimensional patterns obtained from the two methods (Figures 3-A & 4-A).

Surveying the method of using specific kit to remove albumin (Aurum mini protein kit): This kit simultaneously eliminates both albumin and immunoglobulin G from the sample. Examination of one-dimensional protein patterns (Figure 5, columns 2-5) and two-dimensional protein patterns (Fig. 6B) from the output samples of kits and the albumin binding to the resin, which was extracted using HCl (Figure 5 columns 6-9), shows that the kit has great capacity to remove albumin and immunoglobulin (heavy and light chain). However, using this kit nonspecifically removes some amounts of other proteins in binding of albumin and immunoglobulin from the sample (Figure 5 columns 6-9). Thus, to search for biomarkers in the serum using proteomics techniques with respect to the above results, it is better to use a specific removal method for high concentration proteins. Using methods such as trichloroacetic acid precipitation along with the methods based on the columns containing specific resin to remove albumin and immunoglobulin increases the chance to observe the proteins which are naturally in serum binding to albumin and globulins on the protein pattern. As seen in Figure 6, the protein pattern in trichloroacetic acid/acetone precipitation method (6A) is different from in spots 1, 2 and 3 with an approximate molecular weight of 22 kDa and approximate pI 6-8 with the model obtained from kit (6B). Then, the presence or absence and amount of each protein spot were analyzed by Melanie 6 software.

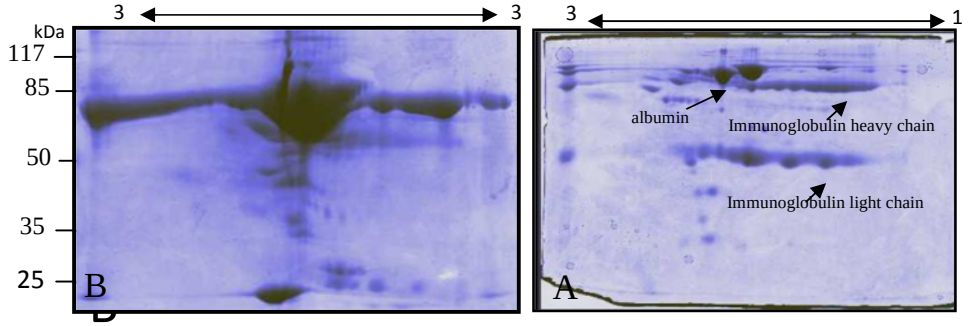

Figure 4. Two-dimensional patterns of serum proteins precipitated with trichloroacetic acid. A 2D pattern of precipitated proteins and B represent the 2D pattern of proteins in supernatant. Proteins were separated on 7 cm IPG (pH 3–10) strips in the first dimension followed by 12% SDS-PAGE

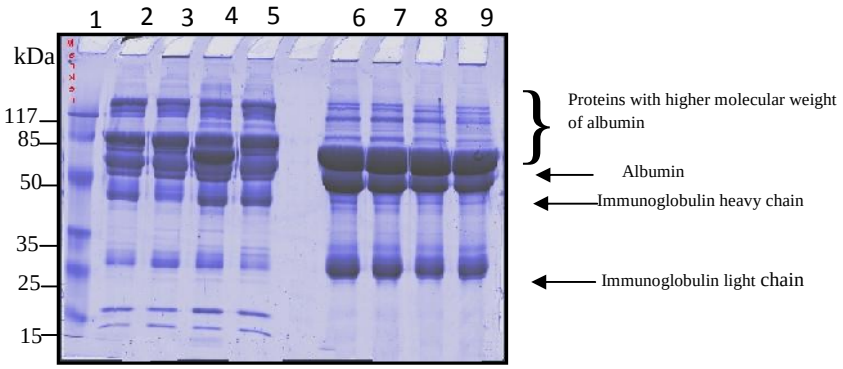

Figure 5. SDS-PAGE pattern of serum proteins precipitated with Aurum mini protein kit. line 1 protein marker, lines 2, 3, 4 and 5 represents the protein pattern after albumin depletion and lines 6, 7, 8 and 9 represent the proteins binded to column.

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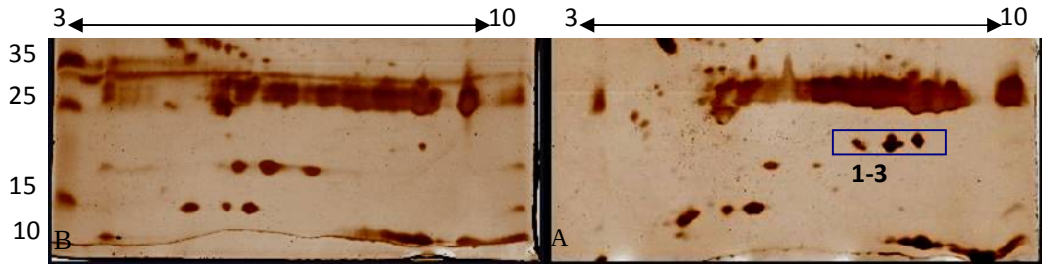

Figure 6. Two-dimensional pattern of serum proteins precipitated with trichloroacetic acid / acetone (A) and kit (B). Proteins were separated on 7 cm IPG (pH 3–10) strips in the first dimension followed by 12% SDS-PAGE

Discussion

In this study we observed that the different methods used to remove albumin and immunoglobulin are able to remove large quantities of albumin from serum, for better detection of low concentration proteins which contain important information on the diagnosis and treatment of diseases. As was observed, the risk of losing samples increases in ammonium sulfate fractionation due to the desalination stage, and more time is needed to conduct it than other methods. However, the use of ammonium sulfate to remove albumin is important, because it cause the least damage to the natural structure of the proteins, especially in many cases that protein activity is important. The study results showed that using trichloroacetic acid/acetone precipitation is a good approach to remove albumin. The comparison of two TCA precipitation methods showed that the second method has eliminated globulin-TCA complex (water soluble) because of using distilled water. Race also used this method to separate globulin from albumin in the serum. Thus, it is better not to use distilled water to examine the sample proteomes and find biomarkers which are mostly proteins with lower molecular weight from globulins group, because globulins TCA complex is soluble in water. Comparison of TCA precipitation method and kit showed that column-based methods and TCA precipitation can be used as parallel methods to remove high concentration proteins from serum. This is a result which cannot be obtained in the studies of Lollo, Chen and Gianazza, due to their use of more limited methods for removal of albumin [4,7,8].

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