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A Simple Method for the Preparation of Human Serum Albumin[1]

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Abstract. A simple method, based on ethanol fractionation, for the preparation of highly purified human serum albumin with a higher yield than that of the conventional ethanol procedures is described. It consists of two purification steps, namely, precipitation of most of the other plasma proteins from a 3-fold diluted plasma with ethanol at 42% concentration, pH 5.75 and –5 °C, leaving over 96% pure albumin in the supernatant, followed by isolectric precipitation of albumin from the supernatant at pH 4.8 and –5 °C. The paste thus obtained was processed to the final albumin solution according to the conventional methods. The yield of the final albumin with a purity of over 99% was equivalent to 29.5 g/l of plasma representing a recovery of over 93%. The possibility of recovering other plasma proteins and the suitability for large scale preparation are also discussed.

Introduction

Ever since *Cohn et al.* [2] followed by *Oncley et al.* [13] developed the cold ethanol procedure for fractionation of plasma into five major fractions, the method for preparing clinical albumin from Cohn fraction V has been predominantly used over the past 30 years throughout the world. The albumin thus prepared was carefully characterized by the Protein Foundation and subsequently approved by the Division of Biologics Standards (DBS) of the National Institutes of Health, now the Bureau of Biologics (BoB) of the Federal Drug Administration (FDA) [12]. In 1962, *Kistler and Nitschmann* [9] simplified the Cohn procedure for the preparation of this plasma derivative but the method still required at least three precipitation steps in order to remove other proteins prior to the recovery of albumin, even if albumin was the only product intended. Because of the ever-increasing demand for this important plasma protein, several alternative methods have been reported, aiming primarily for the preparation of clinical albumin. These include the use of polyethylene glycol for initial fractionation of plasma fol-

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lowed either by electrodecantation [15] or by repeated chromatography on ion exchangers [3], and the method of *Schneider et al.* [16] which involves heating the plasma in the presence of ethanol and a stabilizer followed by precipitation of albumin with polyethylene glycol. However, none of these methods has received wide acceptance by the industrial fractionators in the United States. The reasons for this reluctance to change the method are at least threefold. First, a considerable investment has to be made for the necessary fractionation facilities; second, the question of quality and yield of the product, and the processing scale, and third, the restrictions imposed upon the use of new reagents and precipitants by the BoB.

In our continued search for a new method that would lead to a procedure for those fractionators who are primarily interested in the recovery of clinical albumin, many points of equal importance have to be considered. The method should (1) utilize the existing fractionation facilities and equipment, (2) be simpler than the currently available methods, (3) be amenable to large scale production under aseptic and pyrogen-free conditions, (4) provide a better product in terms of purity and yield and (5) conform to the licensing requirements of the BoB. This report represents the first step toward that goal whereby a simple ethanol procedure to prepare highly purified albumin which appears to meet all these criteria is described.

Materials and Methods

Fresh frozen plasma or cryosupernatant derived from 450 ml of human blood plus 63 ml CPD anticoagulant solution was obtained from the American Red Cross volunteer donors. Ethanol used was USP 95% ethyl alcohol. Acetate buffer [0.8 *M*, pH 4.0) was prepared according to *Cohn et al.* [2]. Sodium caprylate and N-acetyltryptophanate were purchased from the Nutritional Biochemical Corporation. All other chemicals and reagents used were reagent grade.

Centrifugations were carried out with a Sorvall RC-2B centrifuge operating at 12,000 *g* for 1 h at –5 °C. All pH measurements were made at room temperature. Protein mixtures were diluted 5-fold with 0.15 *M* NaCl before measuring pH in order to avoid abnormal readings at high ethanol concentrations. Biuret determinations of protein using bovine albumin as standards were carried out according to the method of *Gornall et al.* [5].

Polyacrylamide gel electrophoresis was carried out according to *Davis* [4] except that the sample gel was omitted. The separating gel contained 7% acrylamide and the staining solution was 1% amido black in 7% acetic acid. Immunoelectrophoresis (IEP) of plasma and various albumin preparations, 3.5% in concentration, against anti-whole serum, was carried out according to *Grabar and Williams* [6]. Quantitation of albumin was carried out by radial immunodiffusion technique (RID) [10] using M-partigen plates, which have a precision of $\leq$ 8.5% coefficient of variation, purchased from Behring Diagnostics, Somerville, N. J. Detection of possible impurities which have the same electrophoretic mobility as albumin was carried out by double diffusion technique [14] using a 10% albumin solution and commercially available antisera against α-globulins such as α-1-antitrypsin, α-1-acid glycoprotein, antithrombin III and α-1-lipoprotein, purchased from Behring Diagnostics.

Prekallikrein activating activity (PKA) of the 25% albumin solution was determined according to the method of *Alving et al.* [1].

Procedure for Isolation of Albumin

A flow chart for the isolation of albumin is shown in figure 1. All precipitation steps were carried out at –5 °C.

Precipitation of Impurities. Threefold diluted plasma was placed in a water bath at –5 °C and the pH was adjusted to 5.6 using acetate buffer. After stirring for 1 h, precooled (–5 °C) 95% etha-

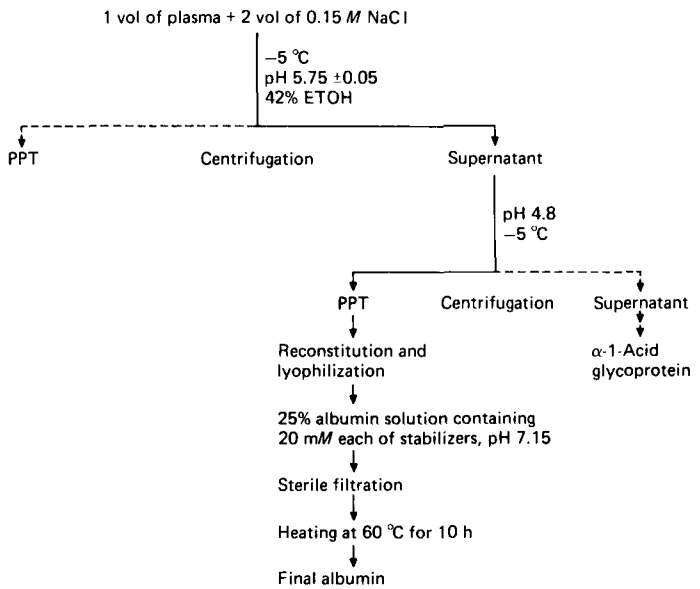


Fig. 1. Flow chart for the isolation of albumin.

nol was added in a thin stream fashion to avoid the high heat of dilution generated by the addition of ethanol to a final concentration of 42% (v/v) with stirring. The temperature of the mixture should not exceed 0 °C. After the ethanol addition the pH became 5.75 ± 0.05 and the mixture was continuously stirred for at least 1 h prior to centrifugation. After centrifugation, the precipitate was discarded and the supernatant was used for further fractionation.

Precipitation of Albumin. The pH of the supernatant was adjusted to 4.8 using acetate buffer. After stirring for 1 h, the mixture was allowed to age without stirring for at least 3 h prior to centrifugation. The precipitate, similar to Cohn fraction V in texture, was the albumin paste to be used for further processing, whereas the supernatant, which is analogous to Cohn fraction V supernatant, could be used for preparing α-1-acid glycoprotein as a byproduct according to the method of *Hao and Wickerhauser* [7].

Preparation of the 25% Albumin Solution. Solutions of various concentrations could be prepared from the albumin paste according to the conventional methods [2, 9]. In brief, in order to prepare the 25% albumin solution, the paste was first reconstituted in 2 vol of distilled H_2O and then lyophilized. This lyophilized powder was then weighed and reconstituted in an appropriate amount of H_2O containing 0.02 *M* each of sodium caprylate and N-acetyltryptophanate followed by adjusting the pH to 7.15 with 2 *N* NaOH and addition of the required amounts of NaCl. This albumin solution, clear yellow in color, was then sterile filtered and pasteurized at 60 °C for 10 h. Tests on the final product including heat stability were carried out according to BoB requirements [12].

Results

In a typical experiment starting from a 300 ml aliquot of fresh frozen plasma pool it required 600 ml of 0.15 *M* NaCl to make the dilution and 1.55 ml of acetate buffer to bring the pH to 5.6, which later became 5.75 after addition of 713 ml of 95% ethanol. Centrifugation gave rise to a precipitate of 46.8 g and a supernatant of 1,518 ml.

Table I. Purification of albumin

	Volume ml	Albumin mg/ml[1]	Total albumin, mg	Protein, mg/ml[2]	Total protein, mg	Purity, %	Recovery, %
Plasma	300	31.5	9,450	67.5	20,250	46.7	100
42% ETOH supernatant	1,518	6.1	9,260	6.3	9,563	96.8	98
Reconstituted albumin paste	167	52.8	8,818	53.2	8,884	99.3	93.3
25% albumin solution	34.7	253.0	8,779	255	8,848	99.2	93

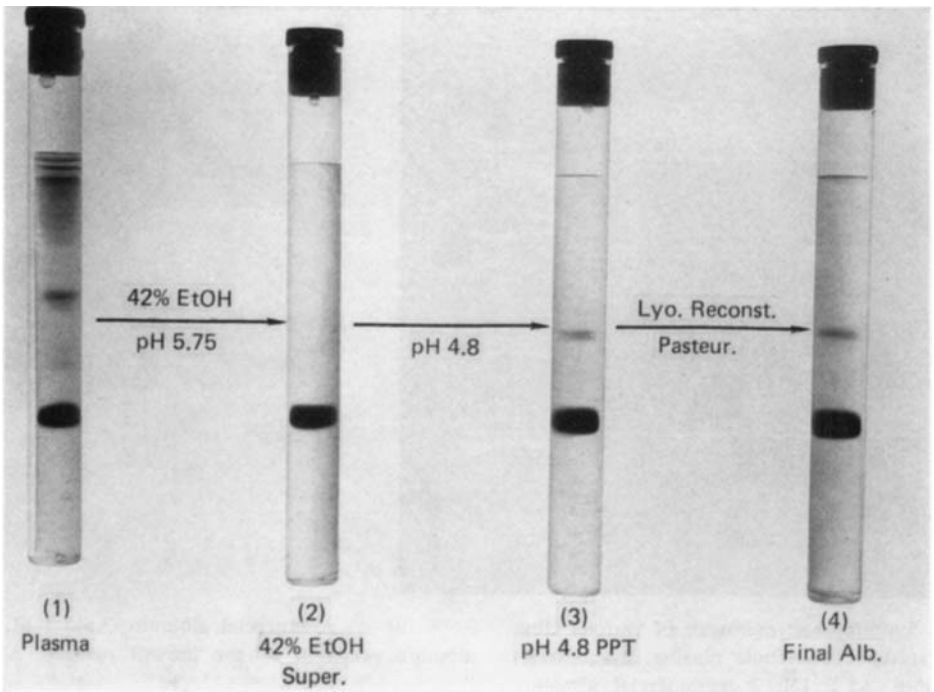
[1] Determined by radial immunodiffusion.
[2] Determined by biuret reaction.

To the supernatant, 0.92 ml of acetate buffer was required to adjust the pH to 4.8. After standing overnight and centrifugation, an albumin paste of 55.6 g and a supernatant of 1,462 ml were obtained. The paste was then reconstituted in 111 ml of H_2O to give a total volume of 167 ml prior to lyophilization whereas the supernatant was used for the recovery of α-1-acid glycoprotein [7].

Upon completion of lyophilization, the albumin powder was dissolved in 33 ml of H_2O containing stabilizers and NaCl, and a final volume of 34.7 ml of 25% albumin solution was obtained. Sterile filtration followed by pasteurization resulted in a clear yellow solution. This solution remained unchanged as determined nephelometrically, after heating at 57 °C for 50 h. Samples which were not properly aged during the isoelectric precipitation step gave rise to a yellow rather than white powder, after lyophilization, and a greenish yellow solution after pasteurization.

The yield of albumin in terms of antigen and protein from each step is summarized in table I. Precipitation with ethanol at 42% removed slightly more than 50% of the total protein, leaving 98% of the albumin in the supernatant. Isoelectric precipitation at pH 4.8 recovered almost all of the albumin in the paste, leaving more soluble proteins, mostly α-1-acid glycoprotein, in the supernatant. The final yield of the 25% albumin solution, having a purity of over 99%, was equivalent to 29.5 g/l of starting plasma which originally contained 31.5 g albumin/l for a final recovery of over 93%. Three plasma aliquots, each derived from a 100-liter plasma pool (cryosupernatant) or 500 donors, were quantitated by RID and their albumin concentration was found to be in the range of 30.2–33.5 mg/ml, which was in good agreement with the albumin concentration in the starting plasma.

Figure 2 shows the polyacrylamide gel electrophoretic results of the purified samples saved from each step during fractionation. The 42% ethanol supernatant revealed essentially only one band (albumin). The reconstituted albumin paste obtained by isoelectric precipitation at pH 4.8 showed a major albumin band as well as a thin dimer band indicating polymerization probably caused by the combination of low pH and



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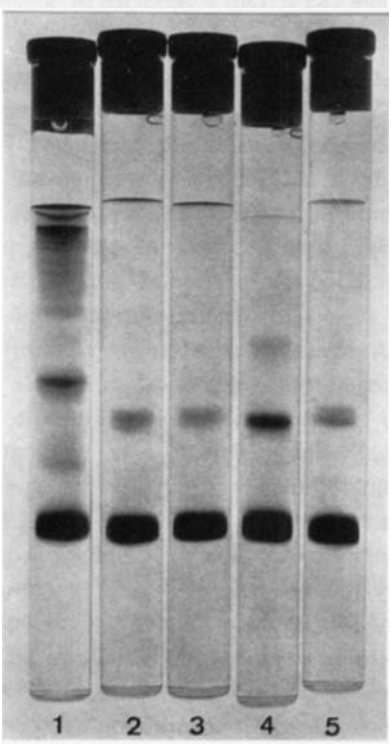

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Fig. 2. Preparation of albumin as illustrated by polyacrylamide-gel electrophoresis. 1 = Plasma, 5 μl; 2 = 42% ETOH supernatant, 25 μl; 3 = pH 4.8 precipitate (5%), 4 μl; 4 = final albumin (25%), 200 μg.

Fig. 3. Polyacrylamide-gel electrophoresis of various albumin preparations. 1 = Plasma, 350 μg; 2 = commercial albumin, 200 μg [2]; 3 = commercial albumin, 200 μg [9]; 4 = commercial albumin, 200 μg [16]; 5 = albumin prepared by the present method, 200 μg.

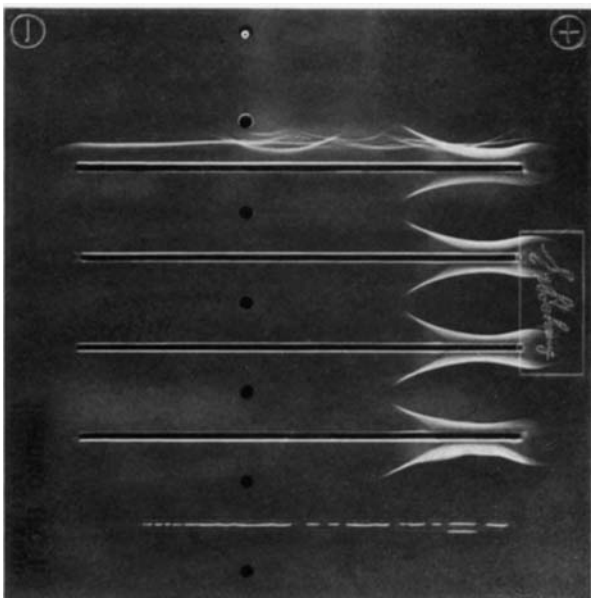

Fig. 4. Immunoelectrophoresis of various albumin preparations. 1 = Whole plasma; 2 = commercial albumin, 3.5% [2]; 3 = commercial albumin, 3.5% [9]; 4 = commercial albumin, 3.5% [16]; 5 = albumin prepared by the present method, 3.5%.

high ethanol concentration. The pasteurized albumin solution was essentially the same as that of the reconstituted albumin paste. Figure 3 shows the electrophoretic results of the albumin prepared by the present method along with three commercial albumin products used for comparison. Figure 4 shows the IEP results of plasma and the same albumin preparations used in gel electrophoresis when developed against antiwhole serum. All four preparations were found to be antigenically homogeneous. In qualitative tests (Ouchterlony) for impurities using various specific antisera, traces of α-1-acid glycoprotein were detected in the albumin prepared by the present method. The concentration of this impurity, however, was below the detectable level by RID which has an assay range of 10–140 mg/100 ml. No PKA was detected in the two preparations tested.

Discussion

In realizing that albumin is probably the most important plasma component currently recovered from the conventional ethanol fractionation systems, it would be ideal to develop a method such as the one reported here whereby albumin could be immediately separated from the rest of the plasma proteins without being subjected to sequential fractionation. However, due to the magnitude of its concentration in plasma, the most reasonable approach for isolating this protein is to work out conditions in terms of protein and ethanol concentrations, pH, ionic strength, which would lead to a sufficiently large difference in solubility between albumin and the rest of the proteins thus permitting a satisfactory separation by precipitation.

In order to achieve better selectivity and less entrapment during precipitation, this method is developed by starting with a 3-fold diluted plasma which becomes 5-fold diluted after ethanol addition, whereas the conventional ethanol procedures [2, 9] give rise to a 2- to 3-fold dilution over plasma when albumin fraction is precipitated. However, this procedure only requires two precipitation steps compared to several precipitation steps required in the conventional procedures, thus resulting in a higher yield, and reducing the processing time and labor considerably. In addition to recovering cryoprecipitate for preparation of antihemophilic factor [18], the present method, although developed primarily for albumin, could also be integrated with the methods for recovery of clinical prothrombin complex [8], antithrombin III [11] and C-1 esterase inhibitor [17], using ion exchangers or affinity adsorption prior to ethanol precipitation. These adsorption processes are often accompanied by the dilution of plasma to some degree, especially if a wash step is required. The present procedure which requires initial dilution of plasma in order to obtain effective separation of albumin from the rest of the plasma proteins could therefore accommodate such dilution. Furthermore, since the first step of this procedure is to precipitate most of the other plasma proteins equivalent to Cohn fractions I, II, III, IV-1 and IV-4, leaving albumin in the supernatant, immune serum globulin should be recoverable from the precipitate according to the conventional ethanol procedure [2, 13] without affecting the yield of albumin. The albumin paste obtained by the isoelectric precipitation from this more diluted ethanol supernatant can be reconstituted and lyophilized without employing an additional clarification step, which is required in the conventional procedures to achieve the required purity and to facilitate sterile filtration.

Although the albumin obtained in the first step (42% ethanol supernatant) is well over 96% pure, as required by the BoB [12], the isoelectric precipitation step is necessary. It not only concentrates albumin from a solution containing 42% ethanol, but removes more soluble proteins, such as α-1-acid glycoprotein, in the supernatant. It has been found necessary to age the precipitate during isoelectric precipitation of albumin to avoid the development of green color after pasteurization. The cause for this color change is unknown and merits further investigation. However, *Kistler and Nitschsmann* [9] postulated that the color change was due to oxidation of traces of hemoglobin to verdoglobin.

Although the present procedure has been developed based on a series of small scale experiments, it appears that the method has the potential of being scaled up since it requires only those steps which have been routinely employed in the conventional ethanol fractionation procedure.

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References

1 Alving, B. M.; Hojima, Y.; Pisano, J. J.; Mason, B. L.; Buckingham, R. E.; Mozen, M. M., and Finlayson, J. S.: Hypotension associated with prekallikrein activator (Hageman-factor fragments) in plasma protein fraction. New Engl. J. Med. *299:* 66–70 (1978).

2 Cohn, E. J.; Strong, L. E.; Hughes, W. I., jr.; Mulford, D. J.; Ashworth, J. N.; Melin, M., and Taylor, H. L.: Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluids. J. Am. chem. Soc. *68:* 459–475 (1946).

3 Curling, J. M.; Berglof, J.; Lindquist, L. O., and Eriksson, S.: A chromatographic procedure for the purification of human plasma albumin. Vox Sang. *33:* 97–107 (1977).

4 Davis, B. J.: Disc electrophoresis. II. Method and application to human serum proteins. Ann. N.Y. Acad. Sci. *121:* 404–427 (1964).

5 Gornall, A. G.; Bardawill, C. J., and David, M. M.: Determination of serum proteins by means of the biuret reaction. J. biol. Chem. *177:* 751–766 (1949).

6 Grabar, P. et Williams, C. H.: Methode immunoélectrophorétique d'analyse de mélanges de substances antigéniques. Biochim. biophys. Acta *17:* 67 (1955).

7 Hao, Y. L. and Wickerhauser, M.: A simple method for the large-scale preparation of alpha-1 acid glycoprotein. Biochim. biophys. Acta *322:* 99–108 (1973).

8 Heystek, J.; Brummelhuis, H. G. J., and Krijnen, H. W.: The large-scale preparation of prothrombin complex. Vox Sang. *25:* 113–123 (1973).

9 Kistler, P. and Nitschmann, H.: Large scale production of human plasma fractions. Vox Sang. *7:* 414–424 (1962).

10 Mancini, G.; Carbonara, A. O., and Heremans, J. F.: Immunochemical quantitation of antigens by single radial immunodiffusion. Immunochemistry *2:* 235–254 (1965).

11 Miller-Andersson, M.; Borg, H., and Anderson, L. O.: Purification of antithrombin III by affinity chromatography. Thromb. Res. *5:* 439–452 (1974).

12 Minimum Requirements: Normal serum albumin (human); 8th ed. (US Department of Health, Education and Welfare, Bethesda 1953).

13 Oncley, J. L.; Melin, M.; Richert, D. A.; Cameron, J. W., and Gross, P. M., jr.: The separation of antibodies, isoagglutinins, prothrombin, plasminogen and beta-1 lipoprotein into subfractions of human plasma. J. Am. chem. Soc. *71:* 541–550 (1949).

12 Ouchterlony, O.: Diffusion-in-gel methods for immunological analysis. Progr. Allergy, vol. 5, pp. 1–78 (Karger, Basel 1958).

15 Polson, A. and Ruiz-Bravo, C.: Fractionation of plasma with polyethylene glycol. Vox Sang. *23:* 107–118 (1972).

16 Schneider, W.; Lefevre, H.; Fiedler, H., and McCarty, L. J.: An alternative method for large scale plasma fractionation for the isolation of serum albumin. Blut *30:* 121–134 (1975).

17 Vogelaar, E. F.; Brummelhuis, H. G. J., and Krijnen, H. W.: Large-scale preparation of human C-1 esterase inhibitor concentrate for clinical use. Vox Sang. *26:* 118–127 (1974).

18 Wickerhauser, M.; Mercer, J. E., and Eckenrode, J. W.: Development of large scale fractionation methods. VI. An improved method for preparation of antihemophilic factor. Vox Sang. *35:* 18–31 (1978).

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