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Purification of human albumin by the combination of the method of Cohn with liquid chromatography

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Abstract

Large volumes of plasma can be fractionated by the method of Cohn at low cost. However, liquid chromatography is superior in terms of the quality of the product obtained. In order to combine the advantages of each method, we developed an integrated method for the production of human albumin and immunoglobulin G (IgG). The cryoprecipitate was first removed from plasma for the production of factor VIII and the supernatant of the cryoprecipitate was fractionated by the method of Cohn. The first precipitate, containing fractions (F)-I + II + III, was used for the production of IgG by the chromatographic method (see Tanaka K et al. (1998) *Brazilian Journal of Medical and Biological Research*, 31: 1375-1381). The supernatant of F-I + II + III was submitted to a second precipitation and F-IV was obtained and discarded. Albumin was obtained from the supernatant of the precipitate F-IV by liquid chromatography, ion-exchange on DEAE-Sepharose FF, filtration through Sephacryl S-200 HR and introduction of heat treatment for fatty acid precipitation. Viral inactivation was performed by pasteurization at 60°C for 10 h. The albumin product obtained by the proposed procedure was more than 99% pure for the 15 lots of albumin produced, with a mean yield of 25.0 ± 0.5 g/l plasma, containing 99.0 to 99.3% monomer, 0.7 to 1.0% dimers, and no polymers. Prekallikrein activator levels were ≤5 IU/ml. This product satisfies the requirements of the 1997 Pharmacopée Européenne.

Key words
- Albumin production
- Industrial chromatography
- Hemoderivate production
- Downstream process
- Plasma derivatives

Introduction

It is well known that the method of Cohn (1) permits the fractionation of large volumes of plasma per batch at low cost compared to other methods. On the other hand, the quality of the product obtained by liquid chromatography is superior (2,3). In order to obtain advantages from both methods, we developed a technologically attractive integrated technique for the production of human albumin from plasma using alcohol fractionation followed by the chromatographic method.

The combination of two or more methods such as precipitation with cold ethanol and liquid chromatography or thermoprecipitation is commonly employed at some plasma frac-

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tionation centers, with some variation in the method but with satisfactory results in terms of product yield and quality (4-7). In 1996 the Bioplasma Division of CSL Limited, Victoria, Australia (8), reported a new process for manufacturing albumin using a combination of Cohn fractionation and chromatographic methods. Plasma was first processed by ethanol precipitation to remove fractions (F) I, II and III according to the method of Cohn (1); the supernatant was then submitted to DEAE-Sepharose FF ion-exchange chromatography followed by CM-Sepharose FF and finally gel filtration on Sephacryl S-200 HR. Albumin produced by the combined method contains very low aluminum levels compared to the product obtained by the Cohn process alone, and the prekallikrein activator (PKA), which is generated by contact activation during processing, is also removed mainly in the chromatographic step (8). A reduction of processing time and the need for less equipment were observed in many cases, without affecting the quality of the product. Thus, this integration of methods is considered to be advantageous also in economic terms (6).

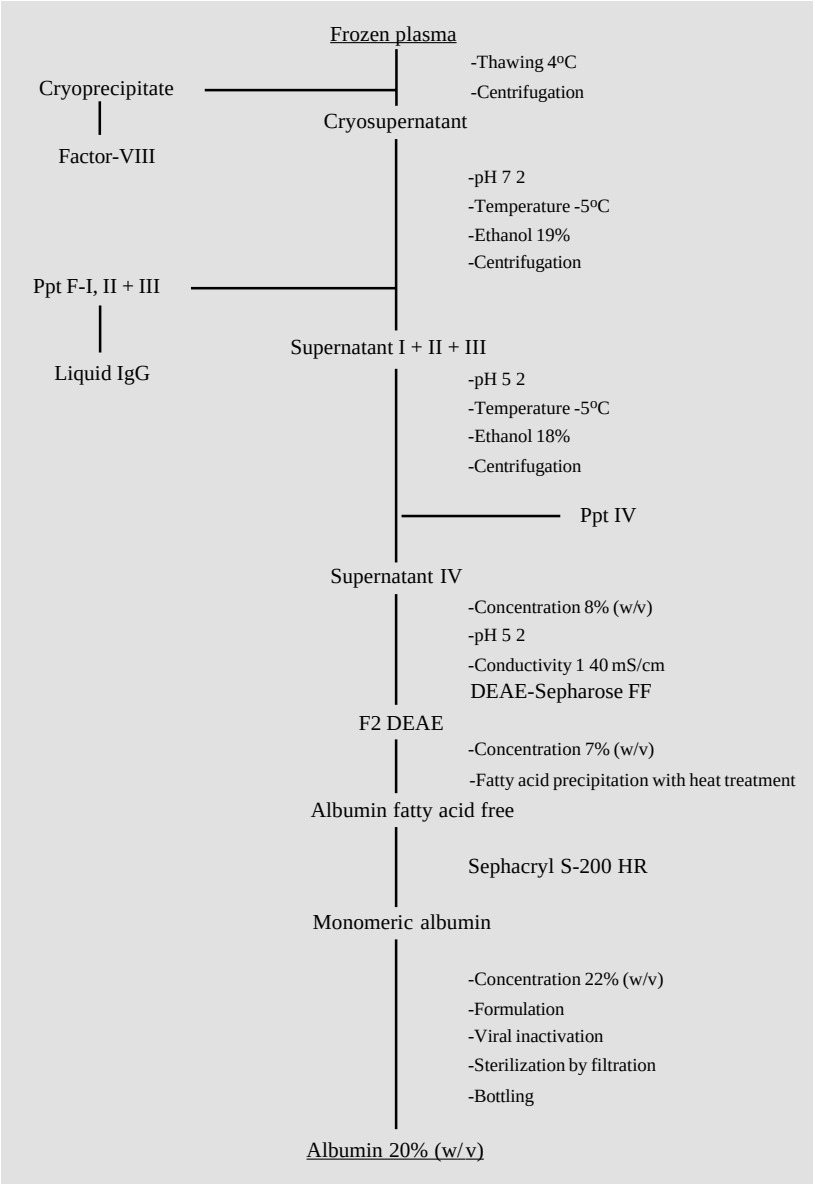

Figure 1 - Flow diagram for the production of human albumin by a combination of the Cohn and liquid chromatography methods. Ppt, Precipitate.

The method summarized in Figure 1 uses two alcohol precipitation steps and two chromatographic steps, i.e., ion-exchange on DEAE-Sepharose FF and gel filtration on Sephacryl S-200 HR.

Material and Methods

One hundred liters of plasma were thawed to 2 to 4ºC and centrifuged at the same temperature to obtain a cryoprecipitate which was stored at -70ºC for later production of factor VIII. The pH of the supernatant of the cryoprecipitate was readjusted to 7.2 with 1 M acetic acid and the mixture was gradually cooled to -5ºC in a stainless steel reactor. Ethanol (95%, w/v) at -30ºC was slowly added with constant stirring until a final concentration of 19% was obtained. The material was left to stand overnight at -5ºC and then centrifuged at the same temperature in a Westphalia BKA-2 centrifuge at a flow of 35 l/h in order to obtain the F-I + II + III precipitate which was used for the production of intravenous immunoglobulin G (IgG) (9,10).

The I + II + III supernatant was adjusted to pH 5.2 with acetate buffer, pH 4.0, containing 0.8 M sodium acetate and 4 M acetic acid, with constant stirring at -5ºC, thus re-

ducing the ethanol concentration to the desired level of 18%. The material was left to stand overnight at -5ºC and then centrifuged at the same temperature at a flow of 35 l/h to remove the F-IV precipitate, which was discarded. The supernatant of the F-IV precipitate was concentrated to 8% (w/v) protein using the Pellicon Cassette System 10,000 NMWL (Millipore, Bedford, MA, USA) and cleared by filtration through a 50-S Zeta Plus membrane (Cuno, Meriden, CT, USA). Conductivity was adjusted to 1.4 mS/cm, pH 5.2, and the liquid chromatography method was used after this step.

Thirteen liters of the sample described above were applied to a PS-370/15 chromatographic column (15 cm in height by 37 cm in diameter) containing DEAE-Sepharose FF ion-exchange gel (Pharmacia Biotech, Uppsala, Sweden) equilibrated with 20 mM acetate buffer, pH 5.2, at 18ºC, at a flow rate of 2 l/min (Figure 2). The albumin eluted from this gel with 25 mM acetate buffer, pH 4.5, presented 96% purity as determined by cellulose acetate electrophoresis. This albumin solution was concentrated about 7% (w/v) using the Pellicon Cassette System 10,000 NMWL (Millipore), its pH was adjusted to 5.2 with 1 M NaOH and its conductivity to 5.0 mS/cm with NaCl. The albumin was then heated to 55ºC for 3 h to precipitate fatty acids, followed by cooling to 4ºC. The precipitate was isolated by centrifugation and the supernatant filtered through 50-S and 90-S Zeta Plus membranes (Cuno). The pH of the albumin solution was adjusted to 6.0 with 1 M NaOH and the material was applied to four PS-370/15 columns (15 cm in height by 37 cm in diameter) connected in series, totalling 60 cm in height, containing Sephacryl S-200 HR filtration gel (Pharmacia Biotech). The columns were equilibrated and eluted with 50 mM NaCl, pH 6.0, at a flow rate of 600 ml/min, at 18ºC to remove the remaining globulins, polymers and protein aggregates (Figure 3). The albumin eluted from the Sephacryl S-200 HR gel was concentrated to 22% (w/v) using the Pellicon Cassette System 10,000 NMWL and submitted to the formulation process using sodium caprylate (0.16 mmol/g protein) to stabilize the product.

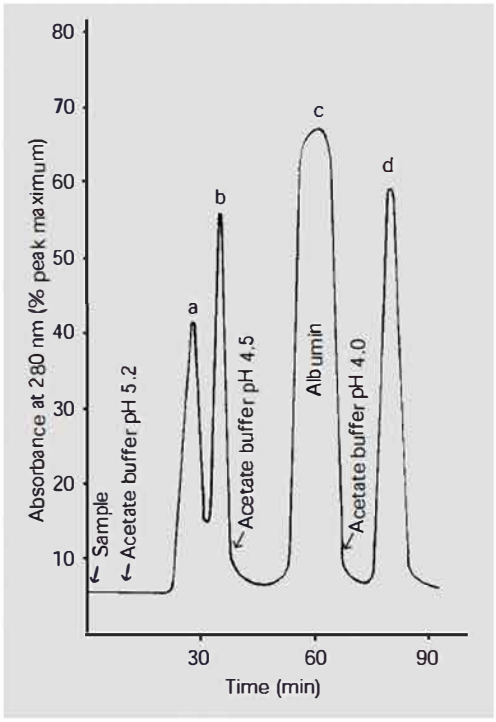

Figure 2 - Elution profile on DEAE-Sepharose FF gel. a, α-, β- and γ-globulins; b, α-globulin and transferrin; c, albumin; d, ceruloplasmin, α_1-chymotrypsin, α_1-antitrypsin and other proteins. Thirteen liters of sample protein at 8% (w/v) concentration were applied to the PS 370/15 chromatography column equilibrated with 20 mM acetate buffer, pH 5.2; peaks a and b were eluted with the same equilibration buffer; peak c was eluted with 25 mM sodium acetate buffer, pH 4.5; peak d was eluted with 150 mM sodium acetate buffer, pH 4.0. The flow rate was 2.0 l/min at room temperature.

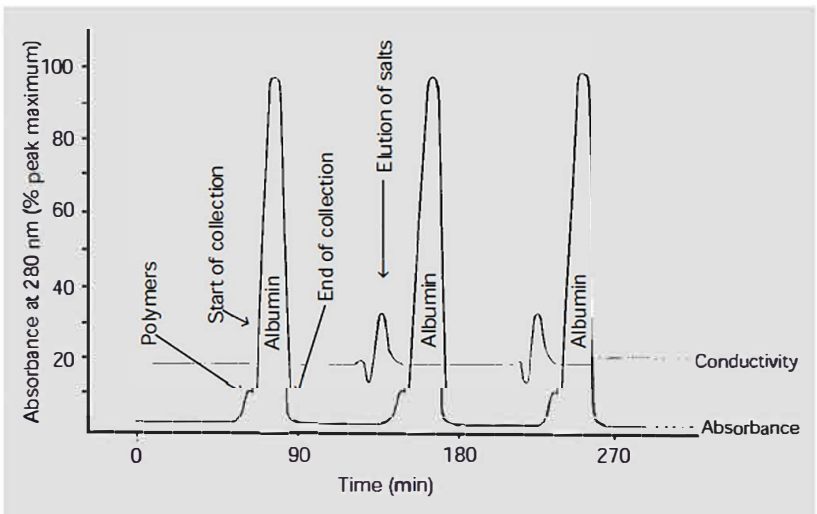

Figure 3 - Elution profile on Sephacryl S-200 HR gel. The sample, 4.0 l of albumin at 6 to 7% (w/v) concentration, was applied to 4 PS 370/15 columns connected in series for each cycle. The columns were eluted with 50 mM sodium chloride, pH 6.0, at a flow rate of 600 ml/min at room temperature.

Viral inactivation was performed by pasteurization at 60ºC for 10 h (11,12). The final product was bottled in a vial containing 50 ml albumin at a concentration of 20%. A schematic presentation of the flow diagram for the production of human albumin is presented in Figure 1.

All the methods for the evaluation of the product were applied according to the guidelines of the Pharmacopée Européenne (13) and to Regulation No. 2.419 of 12/17/1996 of the Brazilian Ministry of Health.

Table 1 - Characteristics of 20% albumin obtained by the method described.

Fifteen lots were analyzed. *Pharmacopée Européenne (13).

	Results	Specifications*
Yield (g/l plasma)	25.0 ± 0.5	–
Protein concentration (w/v)	20.0 ± 1.0%	>95 to <105%
Purity (cellulose acetate electrophoresis)	>99.0%	>95%
Size distribution (Superdex 200)		
Monomers	99.0 to 99.3%	mono + dimers = 95%
Dimers	0.7 to 1.0%	
Polymers	not detected	polymers = 5%
Pyrogens	satisfactory	satisfactory
Abnormal toxicity	satisfactory	satisfactory
Sodium caprylate	0.16 mmol/g protein	–
Prekallikrein activator activity	<5 IU/ml	<35 IU/ml
Aluminum	<50 µg/l	<200 µg/l
pH	6.8 to 7.0	6.7 to 7.3
Sodium	140 ± 5 mmol/l	<160 mmol/l
Potassium	<0.05 mmol/g protein	<0.05 mmol/g protein
Heme (absorbance at 403 nm)	≤0.04	<0.15
Anti-HIV	negative	negative
Anti-HCV	negative	negative
HBS-Ag	negative	negative

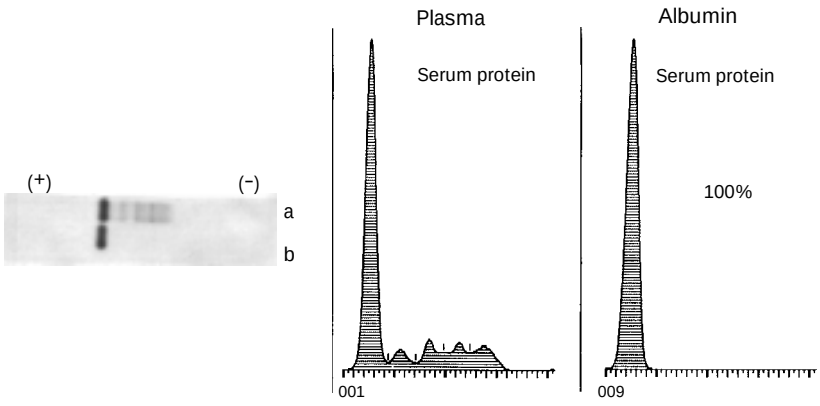

Figure 4 - Cellulose acetate electrophoresis. *a*, Plasma pool; *b*, albumin. The sample volumes were: plasma pool, 25 µl of 10 g/l, and albumin, 25 µl of 30 g/l. Protein was detected with Ponceau S and the data are reported as percent of total densitometer units.

Results and Discussion

The quality control of the albumin of 15 batches produced by a combination of the method of Cohn and liquid chromatography presented highly satisfactory results. The yield calculated for 1 liter of plasma was 25.0 ± 0.5 g and the largest plasma fractionation centers consider this an excellent yield for both the Cohn and chromatography methods. The protein concentration of the albumin solution (final product) was 20.0 ± 1.0% as determined by the biuret method, and purity was more than 99%, as determined by cellulose acetate electrophoresis and by immunoelectrophoresis.

The molecular size distribution of albumin was evaluated by gel filtration using two Superdex 200 HR 10/30 columns (Pharmacia Biotech) in series. The sample volume applied was 200 µl (3%) at a flow rate of 1 ml/min at room temperature, eluted with sodium phosphate buffer, pH 7.0, resulting in the detection of 99.0 to 99.3% monomer, 0.7 to 1.0% dimers and the absence of polymers or aggregates. PKA levels were less than 5 IU/ml as compared to the reference prekallikrein activator of the FDA, and mean aluminum levels were ≤50 µg/l as determined by atomic absorption spectrophotometry. The tests for anti-HIV, anti-HCV and HBsAg antibodies, carried out to guarantee biological safety, were negative. The tests carried out to determine *in vivo* pyrogenicity and toxicity yielded satisfactory results. The characteristics of the final product are reported in Table 1 and illustrated in Figures 4 and 5.

The aim of the present study was to process large volumes of plasma per batch in order to produce human albumin at low cost, with a reduction of processing time to provide a product of high quality in high yields.

The integration of the method of Cohn and liquid chromatography resulted in an attractive alternative in both technological and economic terms. The positive points of this combination of methods were: 1) the use of a small amount of ethanol at a maximum concentration of 19% due to the use of only two precipitation phases with cold ethanol, the 1st for F-I + II + III and the 2nd for F-IV. 2) In the two precipitation steps by the method of Cohn we isolated fibrinogen, γ-globulin, part of α- and ß-globulin, antithrombin III and complement components (1), so that the F-IV supernatant contained high albumin levels of 70 to 75% purity and pH 5.2. Thus, this step provided excellent conditions for the next chromatographic method step. 3) The use of two different processes in the chromatography step, i.e., ion-exchange on DEAE-Sepharose FF (Figure 2) and gel filtration on Sephacryl S-200 HR (Figure 3), resulted in a high quality, pyrogen-free product with a reduction of viruses, as previously reported and validated by Pharmacia Biotech (14), associated with the process of viral inactivation by pasteurization at 60°C for 10 h. This guaranteed the biological safety of the final product. 4) Since the protein level of the F-IV supernatant obtained by the method of Cohn was 2.9%, it was concentrated to 8% (w/v), consequently reducing the volume to half of that of the initial plasma volume. This permitted us to use liquid chromatography equipment of lower capacity, resulting in a reduced consumption of equilibrating buffers and a reduced number of cycles in the production process. Operating time was also reduced from 6 days in the chromatographic method to 4 days in this method, with marked production economy. With the introduction of the albumin fatty acid precipitation step after elution from the DEAE-Sepharose ion-exchange gel, the quality of the product was considerably improved.

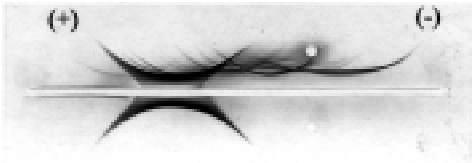

Figure 5 - Immunoelectrophoresis of human plasma (a) and albumin (b) prepared by the method described here. A polyclonal antibody preparation against human plasma was used. The volumes applied to the gel were 2 µl each plasma pool (10 mg/ml) and albumin (30 mg/ml). Protein was detected with light green stain.

The solution was limpid, light yellow to slightly greenish in color, with a visual appearance superior to that of albumin produced entirely by chromatography. Stability was satisfactory after pasteurization and in quarantine tests. The albumin product was 99% monomers and 1.0% dimers and there were no higher aggregates. The mean yield obtained was 25.0 ± 0.5 g/l plasma and the purity of albumin, as determined by cellulose acetate electrophoresis, exceeded 99%, demonstrating the efficiency of the combined method.

We can compare the cost for the production of 200 l of plasma per batch by the original Cohn method, by the whole processing in chromatography method and by the recommended combined method. When the proposed combined method is used the reduction in the expected cost is about 15% compared to the original Cohn method and 30% compared to the chromatography method alone. This combined method was developed on the basis of the availability of raw material in our country and the fact that large-scale production would help to reduce the volume of imported hemoderivatives and consequently their cost.

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