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Large Scale Production of Human Plasma Fractions

Eight Years Experience with the Alcohol Fractionation Procedure of Nitschmann, Kistler and Lergier

P. KISTLER and HS. NITSCHMANN

Central Laboratory of the Swiss Red Cross Blood Transfusion Service, Berne

Introduction

In 1954 the present authors and *Lergier* [8] published a simple method for large scale production of human albumin and gamma-globulin from blood plasma. The procedure makes use of method 10 of *Cohn et al.* [3] and of a suggestion for the extraction of gamma-globulin from Cohn's fraction II and III, made by *Deutsch et al.* [4]. For obvious reasons the method was aimed at obtaining yields as high as possible while achieving purity such as seemed necessary and reasonable.

Since 1954 a total of about 30 000 l of plasma – corresponding to 120 000 blood donations – have been fractionated with this method in the Central Laboratory of the Swiss Red Cross Blood Transfusion Service. During this time the method has been modified slightly and it has been adapted to the requirements of large scale production. The large scale fractionation has been carried out in part in combination with the small pool procedure for the preparation of dried fraction I for clinical use as published by *Nitschman, Kistler and Joss* [7]. Yields and purity of the fractions have been pushed to their practical limit while working time, volumes and alcohol consumption are still remarkably smaller than with Cohn's methods 6 and 9.

The alterations introduced since the first publication and the results attained with the present method are reported.

The Preparation of Albumin

a) *Albumin preparation without previous precipitation of fraction IV.* If the albumin is precipitated directly from the supernatant a according to the description given in 1954 (see scheme in Fig. 1), it will have a purity of 94 % (determined electrophoretically with non preprecipitated material). This degree of purity is quite sufficient if albumin

solutions of 5 to 15 % are to be prepared. These solutions, if stabilized, will withstand the 10 hour pasteurization at 60°C without undergoing alterations to such a degree that the product would not agree with the minimum requirements of the US National Institutes of Health.

Unheated and heated albumin solutions are indistinguishable by electrophoresis, immunoelectrophoresis and in the ultracentrifuge. A quite invisible but measurable increase in turbidity after pasteurization is within the admitted limits. In stability tests the solutions also meet required standards. After storage for several month at room temperature, however some bottles can show trace amounts of flocculant protein material. This has no discernible effect when the solution is transfused.

b) *Albumin preparation with preceding precipitation of a fraction IV.* It is possible to obtain a much purer albumin preparation if, prior to its precipitation, a fraction corresponding to Cohn's IV 1–4 is eliminated by 40 % ethanol at pH 5.8*. This step requires just one more centrifugation and the yield, calculated as pure albumin, is not reduced remarkably. Filtration through clarifying pads is recommended only if highest purity is desired. For routine production of clinical preparations it is unnecessary.

c) *The reworking of the albumin precipitate.* A reprecipitation of the albumin can be omitted with procedures a and b. The alcohol containing paste is suspended in distilled water so that the alcohol content of the suspension will be 10 %. The dry weight of the paste thus must be determined. If Sharples-centrifuges are used the paste will include 60–70 % of its weight as mother liquor which is 40 % alcohol. The final suspension should not contain more than 10 % protein. If necessary it is diluted with the proper amount of 10 % alcohol. After equilibration at pH 4.6, followed by centrifugation to eliminate the insoluble proteins, the solution must be clarified. We find Seitz** carbon black filter pads in combination with Filtrox-D7*** pads very satisfactory. The clarified solution is freeze dried after suitable dilution with water, depending on the efficiency of the freeze drying equipment.

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* For the preparation of 4% albumin solution it is possible to use instead of these conditions the proceeding given by *Hink et al.* [5a] for "Plasmanate"-preparation: pH 5.2, 19% ethanol.

** «Seitz»-Werke, Kreuznach, Germany.

*** Filtrox-Werke, St. Gallen, Switzerland.

d) *Preparation of the final albumin solutions.* The lyophilized albumin may be dissolved to solutions of from 4 to 25 %. After sterile filtration and addition of stabilizers the solutions are heated 10 hours at 60°C for hepatitis virus inactivation. We prepare two types of solutions, one with 15 % and one with 4 % albumin.

The 15 %, low salt, albumin solution (Na^+ ca. 50 mMol/liter) is stabilized with sodium mandelate (0.017 M) and sodium caprylate (0.017 M). The pH is adjusted to about 7. The solution contains 3 % glucose for isotony. 70 ml are oncotically equivalent to 250 ml of plasma with a protein content of 7.5 %.

The 4 % albumin solution contains Na^+, K^+ and Cl^- ions in physiological concentration. It is stabilized with sodium caprylate only (0.008 M). Ascorbic acid (0.24 g/l) is added as an antioxidant to avoid any color change during pasteurization. The color change probably is due to oxidation of traces of haemoglobin to verdoglobin.

The Preparation of Gamma-Globulin

a) *Subfractionation of precipitate A.* In our paper of 1954 [8] no precise values for ionic strength were given because they cannot be measured directly. A calculation of ionic strength in solutions of such complex composition is not easily done. Nevertheless, careful adjustment of ionic strength is of great importance during the fractionation of precipitate A. Slight deviations in ionic strength will influence yield and purity of the gamma-globulin remarkably. A rigorous standardisation of the processing is therefore indispensable. With optimal ionic strength the alcohol concentration of the extraction step can be lowered from the originally suggested 17 % to 12 %.

At present our procedure is as follows:

Each kilogram of precipitate A paste is suspended and homogenized in 10 l of water at 0°C. The pH is then adjusted to 4.8 ($\pm$ 0.05) by adding a pH-4 buffer composed of one volume of 0.05 M Na_2HPO_4 and 6 volumes of 0.05 M acetic acid. 2.5 ($\pm$0.4) liters are necessary. The ionic strength is then increased by adding pH-4.8 buffer composed of 1 volume of 0.05 M Na_2HPO_4 and 1.65 volumes of 0.05 M acetic acid. The amount of this buffer needed is 4.85 l minus the volume of pH-4 buffer added previously for pH adjustment.

For the fractionation the pH is raised to 5.1 ($\pm$ 0.04) by adding pH-6.2 buffer made up of 1 volume of 0.05 M Na_2HPO_4 and 0.833 vol. of 0.05 M acetic acid. Approximately 4.5 l of this buffer will be

necessary. Then the ionic strength is adjusted by adding a pH 5.1 buffer, composed of 1 vol. of 0.05 M Na_2HPO_4 and 1.25 vol. of acetic acid; the amount needed is 4.9 l minus the volume of pH 6.2 buffer. Finally the suspension is diluted with 9.7 l of water.

The total volume of solvent added is now 19.45 l for each kg of precipitate A.

The Na_2HPO_4 molarity is about 0.007. If we assume that at pH 5 the electrolyte contains solely Na^+, $H_2PO_4^-$ and Ac^- ions, the solvent added has an ionic strength of about 0.014. The alcohol concentration now is brought to 12 %. No further adjustment of the ionic strength is made.

The extract (supernatant b in Fig. 2) contains a certain amount of albumin which, at pH 7.2 and this very low ionic strength, forms a complex with gamma-globulin. If the gamma-globulin is precipitated by 25 % alcohol under these conditions the albumin also will go into the precipitate, though it is very soluble in the free state. Consequently it is important before precipitation to raise the ionic strength in supernatant b with NaCl to 0.03–0.04. Then the pH is brought to 7.2 and the alcohol to 25 %. The coprecipitation of albumin thus can be avoided and an electrophoretically pure gamma-globulin is obtained.

b) *Gamma-globulin solution for clinical use.* Until the present the lyophilized gamma-globulin has been dissolved to the traditional 16 % solution for intramuscular use. To achieve isotony and to improve solubility and stability as well, glycin (0.3 Mol/l) is added. Merthiolate (0.1 g/l) serves as an antibacterial agent.

Work is presently in progress to prepare a gamma-globulin solution for intravenous use. It is based on the results of an investigation by *Barandun, Kistler, Jeunet and Isliker* [1] on the incompatibility found in certain individuals to intravenously administered normal gamma-globulin.

The Preparation of Fraction I

In 1957 we published with *Joss* [7] a very simple technic of preparing fraction I for clinical use from small pools. This technic also allows large scale production and has proved very satisfactory throughout the years.

The clue to the simplicity of our technic lays in the omission of any filtration of the fibrinogen solution. The fraction I preparation is being used as fibrinogen and as antihemophilic globulin as well,

with very good clinical results [5]. One unit of our dried fraction I derived from two blood donations contains approximately 1 g of clottable fibrinogen and 1 g of other plasma proteins. After dissolving to a volume of 125 ml it contains the AHG of 500 ml fresh plasma, achieving a 4 fold concentration. It seems dubious to us that it is worthwhile to purify the AHG further with one of the recently published methods when the inevitable losses in yield are considered. Clinical experiences seem to indicate that the combination of fibrinogen and AHG with other factors of the clotting system in a nearly native state is, in certain cases, more effective than would be expected for purified fractions. Moreover, the small-pool-fraction I comprises only a small risk of hepatitis.

Occurrence and Isolation of other Plasma Proteins

a) *Transferrin.* Transferrin is almost quantitatively located in fraction IV. It can be easily isolated from the fraction, preferably by the rivanol method [6].

b) *Plasminogen.* This proenzyme has been isolated from precipitate B with the method of *Nitschmann, Schlunegger and Schneider* [9].

c) *Pituitary Gonadotropic Hormone.* Working with plasma of women in the menopause we found this hormone activity to be concentrated in the albumin fraction obtained after elimination of fraction IV [2].

d) *Clotting Factors VII, IX and X.* The VII-IX-X complex is located mainly in suspensions of precipitate B, from which it may be concentrated by adsorption on $BaSO_4$.

e) *Prothrombin.* Most of the prothrombin activity is present in precipitate B.

Yields and Purities

The figures for yields and purities reported in 1954 were based on a limited number of 10-liter-runs. The values, therefore, varied in a rather wide range.

We now can give the results of a statistical evaluation of about 150 100-liter-runs carried out during the years 1960–1962. See table I.

It may be noted that the yield of gamma-globulin is definitely lower when prepared from a supernatant of fraction I instead of from whole plasma. The losses cannot be explained merely by the inclusion of mother liquor in the fraction-I-paste. It seems that when

fraction I is precipitated (pH 7.2, 8% ethanol) some gamma-globulin is held back by protein – protein interaction with the fibrinogen.

The conditions for the fractionation steps presently applied at the Red Cross Laboratory are summarized in the following schemes (Fig. 1 and 2).

TABLE I

Fraction	Yield* % of sum of plasma proteins A ± m	σ	% of total amount of fraction present in plasma	Purity** in %
Albumin prepared without precipitation of fraction IV	47.7 ± 0.55	3.9	91.6	93–95
Albumin prepared with precipitation of fraction IV				97–98
Gamma-globulin after increasing $\Gamma/_2$ to 0.03 for precipitation	9.82 ± 0.12	1.0	89.2	99
Gamma-globulin from plasma devoid of fraction I	8.62 ± 0.22	1.17	79.5	99
Clottable fibrinogen in «small pool» fraction I	3.4 ***		85	† 47

Explanation Tab. I

* *Yields.* First column: The standard deviation σ was calculated as $\sigma = \sqrt{\frac{\Sigma \Delta^2}{n}}$

and the average error as $m = \pm \frac{\sigma}{\sqrt{n}}$

Second column: To calculate these values we assumed [10] that the proteins of unfractionated plasma consist of

52% albumin
11% gamma-globulin and
4% fibrinogen

These values were set as 100%. The % figures given for albumin and gamma-globulin were calculated after substracting the accompanying proteins from their weight.

** *Purity* was determined by paper electrophoresis. The strips were cut and the bands eluted. The concentration of the solutions was determined colorimetrically.

*** Analysis were made on randomly selected bottles from every 100-liter-run.

† Percentage of clottable proteins of the total proteins in fraction I (mean value from 100 analysis).

Fig. 1

Fractionation scheme: plasma ⟶ albumin

Plasma: — fresh or reconstituted dry plasma, ACD – or EDTA-stabilized 5,5% protein.

8–10%* ethanol**
pH 7.2 ± 0.2
– 3° C
5% protein

centrifugation

⟶ precipitate: *fraction I*, containing fibrinogen (ca. 50% clottable protein) and AHG. "Small pool" process: one unit of fraction I from plasma from only two blood donations. Distributed in lyophilized form.

supernatant
globulins and
albumin

19% ethanol
pH 5.85 ± 0.05
– 5° C
4.5% protein

centrifugation

⟶ *precipitate A*, containing all of the gamma-globulins and part of the α- and β-globulins. (further processing see Fig. 2).

supernatant a
composition:
80% albumin
20% globulins

40% ethanol ***
pH 5.85 ± 0.05
– 8° C
~ 2.5% protein

centrifugation

⟶ *precipitate IV*, containing α- and β-globulins, transferrin, part of ceruloplasmin and little albumin.

* Footnotes see pag. 423.

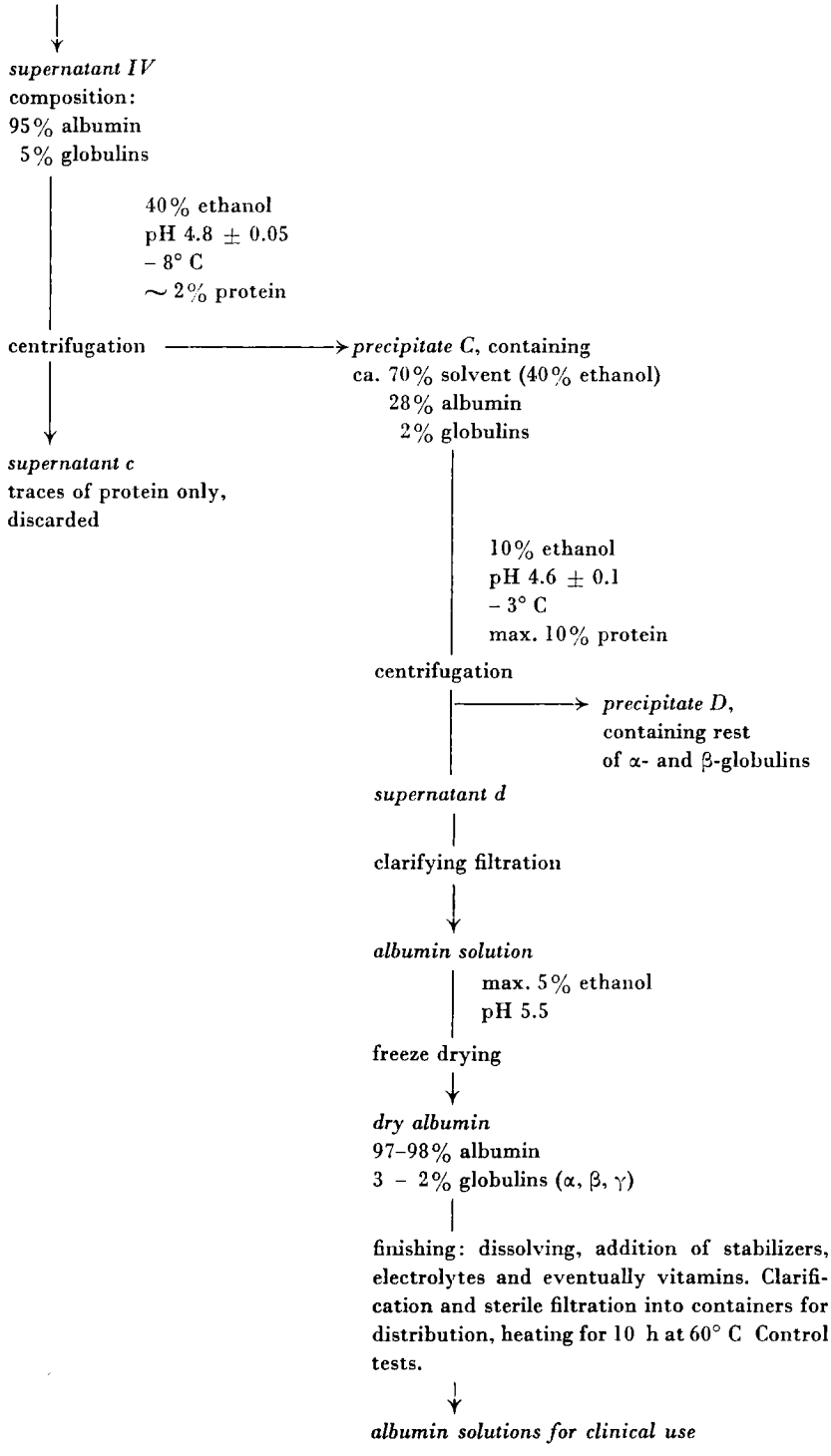
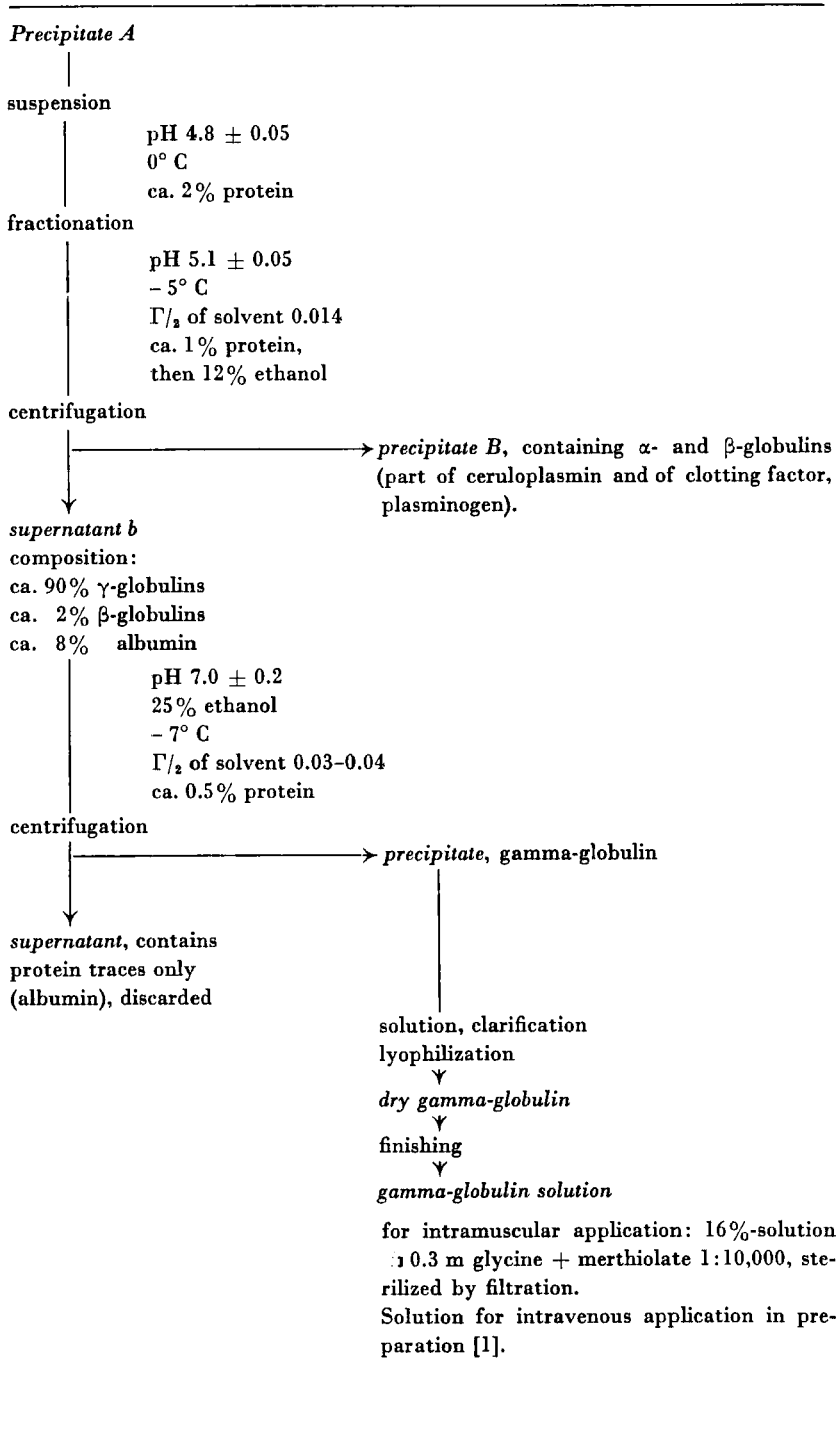

supernatant IV
composition:
95% albumin
5% globulins
40% ethanol
pH 4.8 ± 0.05
– 8° C
~ 2% protein
centrifugation
precipitate C, containing
ca. 70% solvent (40% ethanol)
28% albumin
2% globulins
supernatant c
traces of protein only,
discarded
10% ethanol
pH 4.6 ± 0.1
– 3° C
max. 10% protein
centrifugation
precipitate D,
containing rest
of α- and β-globulins
supernatant d
clarifying filtration
albumin solution
max. 5% ethanol
pH 5.5
freeze drying
dry albumin
97–98% albumin
3 – 2% globulins (α, β, γ)
finishing: dissolving, addition of stabilizers, electrolytes and eventually vitamins. Clarification and sterile filtration into containers for distribution, heating for 10 h at 60° C Control tests.
albumin solutions for clinical use

Fig. 2

Fractionation scheme: precipitate A ⟶ gamma-globulin



Footnotes to Fig. 1:

* %Vol., measured at cold room temperature.

** If fraction I is not wanted precipitate A can be precipitated directly from the plasma under the appropriate conditions. This always will apply when the plasma is not fresh. Fraction I then goes into precipitate A.

*** The elimination of precipitate IV may be avoided by adjusting directly to the conditions for the precipitation of the albumin: most of the proteins of fraction IV then will be found in precipitate D. The purity of the albumin will be a few percent lower.

Summary

The modifications put on the alcohol fractionation procedure of *Nitschmann, Kistler* and *Lergier* [8] since 1954 are communicated. The yields and purities realised were calculated from the results of about 150 fractionation runs, 100 l of plasma each. The actual method allows the isolation of 48% of the total plasmaproteins as albumin (98% pure in electrophoretic analysis), 9,8% as γ-Globulin (practically 100% pure) and 3,4% as clottable fibrinogen. A scheme summarizes the details of the entire procedure.

Résumé

Les modifications apportées depuis 1954 à la méthode de fractionnement de *Nitschmann, Kistler* et *Lergier* [8] sont décrites. Le rendement et la pureté des differents fractions ont été calculées à partir des résultats de 150 fractionnements de 100 litres chacun. La méthode actuelle permet d'obtenir 48% des protéines plasmatiques totales sous forme d'albumine (degré de pureté électrophorétique de 98%), 9,8% sous forme de γ-globuline (degré de pureté pratiquement de 100%) et 3,4% sous forme de fibrinogène coagulable. Un schéma détaillé résume l'ensemble du procédé de fractionnement.

Zusammenfassung

Die seit 1954 vorgenommenen Veränderungen der Alkoholfraktionierungsmethode nach *Nitschmann, Kistler* und *Lergier* [8] werden dargelegt. Die mit der Methode praktisch erzielten Ausbeuten und Reinheiten wurden aus den Ergebnissen von rund 150 100-Liter-Plasma-Ansätzen ermittelt: Nach dem heutigen Verfahren lassen sich etwa 48% der totalen Plasmaproteine als Albumin (ungefähr 98% elektrophoretisch rein), 9,8% als γ-Globulin (praktisch 100prozentig rein) und 3,4% als gerinnbares Fibrinogen gewinnen. Das ganze Fraktionierverfahren ist in Form eines detaillierten Schemas zusammenfassend dargestellt.

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Authors' address: Dr. P. Kistler, Zentrallaboratorium, Blutspendedienst SRK, Wankdorfstrasse 10, *Berne* (Switzerland)