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High quality human immunoglobulin G purified from Cohn fractions by liquid chromatography

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Abstract

In order to obtain intravenous immunoglobulin G (*iv* IgG) of high quality from F-I+II+III or F-II+III pastes prepared by the Cohn method, we developed a chromatography process using ion exchange gels, Q-Sepharose FF and CM-Sepharose FF, and Sephacryl S-300 gel filtration. Viral inactivation was performed by incubating the preparation with pepsin at pH 4.0 at 35ºC for 18 h. The characteristics of 28 batches produced by us were: yield 4.3 ± 0.2 g/l plasma, i.e., a recovery of 39.1 ± 1.8%; IgG subclasses distribution: IgG_1 = 58.4%, IgG_2 = 34.8%, IgG_3 = 4.5% and IgG_4 = 2.3%; IgG size distribution was 98.4% monomers, 1.2% dimers and 0.4% polymers and protein aggregates; anticomplement activity was less than 0.5 CH_{50}/mg IgG, and prekallikrein activator activity (PKA) was less than 5 IU/ml. These characteristics satisfied the requirements of the European Pharmacopoea edition, and the regulations of the Brazilian Health Ministry (M.S. Portaria No. 2, 30/10/1998).

Key words
- Immunoglobulin G purification
- Hemoderivative purification
- Chromatographic procedures
- High quality immunoglobulin G
- Downstream process

Albumin is produced by the method of Cohn (1) at several human plasma fractionation centers in South America. Plasma is submitted to precipitation steps to separate albumin-rich fractions from fractions containing IgG. The fractions containing IgG, known as F-I+II+III, can be precipitated as a paste in a single step or separately in two steps, first yielding F-I and then F-II+III. Most of these precipitates are not processed to obtain IgG owing to technological difficulties of purification and stability. Current requirements for intravenous IgG are for an intact molecule, low anticomplementary activity and preferably in a liquid formulation, although lyophilized intravenous IgG has been available for many years (2,3). These requirements need a modern methodology such as the use of chromatographic processes for purification of IgG. In order to obtain IgG of excellent quality from the F-I+II+III or F-II+III pastes produced by the method of Cohn, we developed a process in which these precipitates can be submitted to chromatography using three kinds of gels: two ion exchange gels (Q-Sepharose FF and CM-Sepharose FF) and gel filtration (Sephacryl S-300 HR). In a pilot study, 28

IgG batches were purified from 2 liters of plasma per batch. The first 16 IgG batches were obtained from F-I+II+III Cohn pastes and the remaining 12 from F-II+III Cohn pastes.

The F-I+II+III paste (230 g) was dissolved in cold 0.85% saline solution, the pH was adjusted to 7.2 with 0.5 M acetic acid, and the final volume was completed to 2 liters, the same as the initial plasma volume. Conductivity was 10.0 ± 0.5 mS/cm. This F-I+II+III solution was cooled to -3°C and cold ethanol (-30°C) was slowly added to a final ethanol concentration of 5% (v/v) to precipitate fibrinogen (F-I). F-I was removed by centrifugation at 4,000 rpm for 10 min, at -3°C (Sorvall RC 3B Plus, Dupont, Wilmington, DE, USA) and its supernatant F-II+III was diafiltered by tangential ultrafiltration with a Millipore Pellicon Cassette 30 kDa NMWL PLGC to remove ethanol and to reduce conductivity to 1.4 mS/cm. The final volume of the F-II+III solution was the same as that of the initial plasma (2 liters), and pH was adjusted to 6.0 with 0.5 M acetic acid. This solution was then immediately taken to a cold chamber at 4°C, so that the euglobulin precipitate could be obtained and left to stand overnight at 4°C. Euglobulin was removed by centrifugation at 4,000 rpm for 10 min at 4°C. The protein solution had a volume of 1950 ml, pH 6.0, and conductivity of 1.4 mS/cm, ideal conditions for liquid chromatography (4,5). See flow diagram in Figure 1.

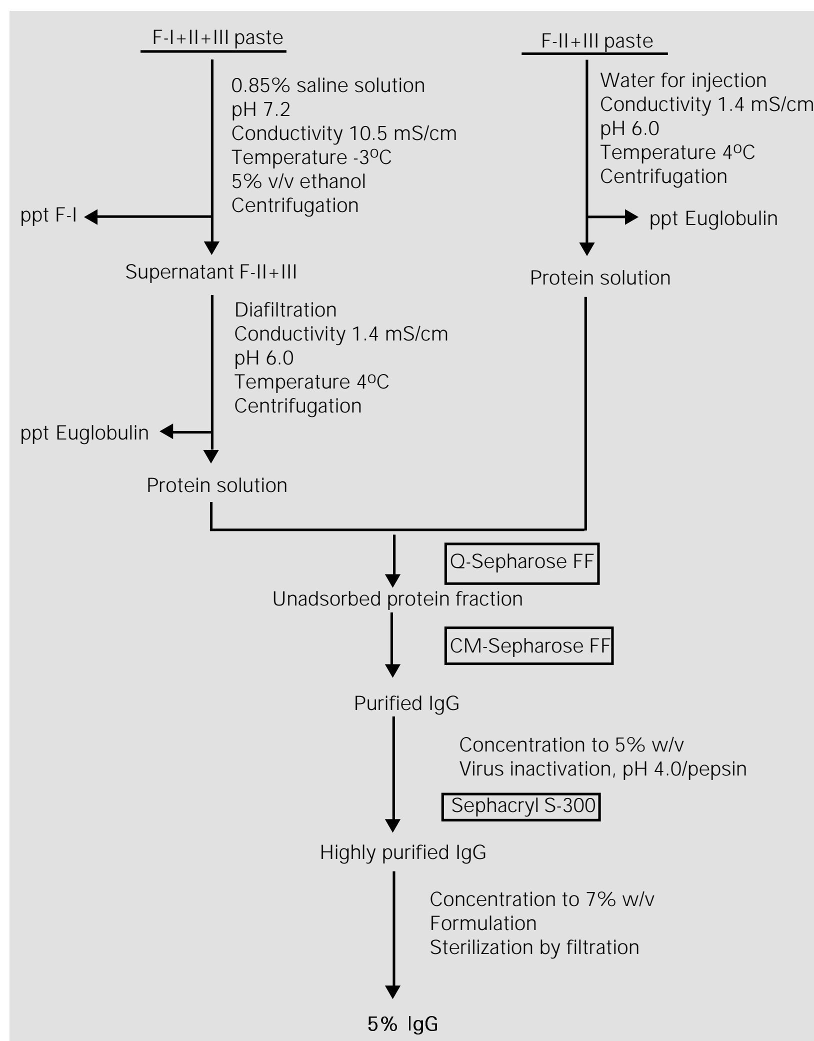

Figure 1 - Flow diagram for the purification of IgG suitable for intravenous administration.

The F-II+III paste (200 g) was dissolved in cold water for injection at a volume sufficient to reach 1.4 mS/cm conductivity, about 1600 ml. The pH was then adjusted to 6.0 with 0.5 M acetic acid and the solution was immediately taken to a 4°C cold chamber to precipitate euglobulin. The precipitated euglobulin was removed by centrifugation at 4,000 rpm for 10 min, at 4°C. The supernatant was submitted to liquid chromatography (4,5). See flow diagram in Figure 1.

Chromatographic procedure (4,5): an XK-50/30 column (Pharmacia, Uppsala, Sweden) containing 320 ml of anion exchange Q-Sepharose FF gel was connected to another XK-50/30 column containing 160 ml cation exchange gel, CM-Sepharose FF. Both of them were previously equilibrated with 20 mM sodium acetate, pH 6.0, and buffer with 1.4 mS/cm conductivity. The gamma globulin solution was applied in three cycles at a flow rate of 40 cm/h. The proteins not relevant to the present study, such as IgA, IgM, transferrin, etc, were adsorbed to the first column, Q-Sepharose FF, and IgG was adsorbed to the second column, CM-Sepharose FF. At the end of the first cycle,

the first column was disconnected from the second using a 3-way valve. The contaminant proteins adsorbed on Q-Sepharose FF were eluted with 0.5 M sodium acetate buffer, pH 7.0, with 25 mS/cm conductivity. The column was reequilibrated with buffer and connected again to the second column in order to start the 2nd cycle, and so on. In this procedure, IgG was adsorbed to the second column on CM-Sepharose FF and eluted with 1 M of NaCl in the three cycles and then immediately concentrated to 5% (w/v) through a Pellicon Cassette System, 30 kDa NMWL PLGC, and the volume obtained was 220 ml of IgG. The IgG solution was submitted to viral inactivation, pH was adjusted to 4.0 with 0.5 M HCl, and conductivity to 5.0 mS/cm. Pepsin was then added (0.1 mg/g of IgG) and the preparation was heated at 35ºC for 18 h (6,7). After viral inactivation the IgG solution was cooled to 20ºC, the pH was adjusted to 6.0 with 0.5 M NaOH and the solution was applied to an XK-26/40 column containing 200 ml of Sephacryl S-300 HR filtration gel to remove aggregated IgG and pepsin, and processed over five cycles. A solution of 0.15 M NaCl was used to equilibrate the column gel and to elute IgG at a flow rate of 45 cm/h. The IgG solution obtained was concentrated to 7%. The final step was a sterilized filtration through a 0.22-µm Millipore membrane (5). One part of IgG was freeze-dried and the other was stored at 4ºC for more than eight months without any change in the product. All gels were regenerated with 0.5 M NaOH and stored in 0.1 M NaOH until the next use, as recommended by Pharmacia (8,9).

The characteristics of the IgG satisfied the requirements of the European Pharmacopoea (10) and the regulations of the Brazilian Health Ministry (11). Protein concentration was determined by the Biuret method and pH was measured after diluting to 1% protein concentration in 0.9% sodium chloride solution. IgA and IgM were measured by the micro-Ouchterlony method and the material was purified by cellulose acetate electrophoresis. Anticomplement activity was determined by the method described in the European Pharmacopeia (10). Prekallikrein activity was determined using S2302, the chromogenic substrate of Chromogenix (Mölndal, Sweden) and the PKA international standard reference 82/530 was obtained from the National Biological Standard Board (Hertfordshire, UK). The molecular weight distribution was analyzed on an XK-16/70 column loaded with Superdex 200 HR gel using high molecular weight and low molecular weight calibration kits (Pharmacia). The IgG subclasses were determined by radial immunodiffusion on Bindarid plates (The Binding Site Inc., San Diego, CA, USA). The presence of anti-A and anti-B hemagglutinins was determined by the indirect Coombs method (see Table 1). The chromatographic methods for virus removal/inactivation during the process is currently a well-established technique for the purification of plasma derivatives (12) which, in

Table 1 - Characteristics of an IgG solution suitable for intravenous administration.

N = 28. ND = Not detected. *European Pharmacopoeia (10) and regulation of the Brazilian Health Ministry – Portaria No. 2 (11).

Analysis	IgG	Specifications*
Protein concentration	5.0 ± 0.2%	>90 to <110%
pH	5.0-5.5	4.0-7.4
Purity (electrophoresis)	>99%	>95%
IgA	ND	–
IgM	ND	–
Anti-A hemagglutinin	≤1:8	<1:64
Anti-B hemagglutinin	≤1:8	<1:64
Anticomplement activity	<0.5 CH_{50}/mg IgG	<1 CH_{50}/mg IgG
PKA determination	<5.0 IU/ml	<35 IU/ml
Size distribution		
Monomers	98.4 ± 0.5%	monomers + dimers = 90%
Dimers	1.2 ± 0.2%	
Polymers	0.4 ± 0.2%	polymers <3%
IgG subclasses		
IgG_1	58.4%	subclass distribution similar to plasma
IgG_2	34.8%	
IgG_3	4.5%	
IgG_4	2.3%	
Yield	4.3 ± 0.2 g/l plasma	–
Stability (heating to 57ºC/4 h)	no jelling	no jelling

combination with a pH 4.0/pepsin treatment for viral inactivation (6,7), provides a very safe product. This purification process can be easily programmed for large-scale production.

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