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A chromatographic method for the production of a human immunoglobulin G solution for intravenous use

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Abstract

Immunoglobulin G (IgG) of excellent quality for intravenous use was obtained from the cryosupernatant of human plasma by a chromatographic method based on a mixture of ion-exchange, DEAE-Sepharose FF and arginine Sepharose 4B affinity chromatography and a final purification step by Sephacryl S-300 HR gel filtration. The yield of 10 experimental batches produced was 3.5 g IgG per liter of plasma. A solvent/detergent combination of 1% Tri (n-butyl) phosphate and 1% Triton X-100 was used to inactivate lipid-coated viruses. Analysis of the final product (5% liquid IgG) based on the mean for 10 batches showed 94% monomers, 5.5% dimers and 0.5% polymers and aggregates. Anticomplementary activity was 0.3 CH_{50}/mg IgG and prekallikrein activator levels were less than 5 IU/ml. Stability at 37ºC for 30 days in the liquid state was satisfactory. IgG was stored in flasks (2.5 g/flask) at 4 to 8ºC. All the characteristics of the product were consistent with the requirements of the 1997 Pharmacopée Européenne.

Key words
- Immunoglobulin G production
- Hemoderivate production
- Intravenous gamma globulin production
- Industrial chromatography
- Downstream process

Introduction

Commercially available liquid or lyophilized immunoglobulins G (IgG) are produced from pooled human plasma from a large number of donors, usually more than one thousand, so that a wide variety of antibodies will be present in the product (1). Several production processes are employed, most of them based on the method of Cohn (2), i.e., fractionation with cold ethanol, with polyethyleneglycol (PEG) precipitation (3) or purification by ion-exchange and gel filtration chromatography (4,5). The IgG isolated from human plasma in the 1940's and 1950's by the method of fractionation with cold ethanol of Cohn and Oncley (2,6,7) was suitable for intramuscular use and could not be administered intravenously because of undesirable effects due to the modifications in the IgG molecule, such as aggregates resulting from the fractionation process with ethanol and other agents which increased its anticomplement activity (8,9).

Thus, the major goal of IgG producers was to develop methods that would guarantee intravenous tolerance, eliminating or preventing the aggregation of molecules without affecting the activity of the antibodies present in the IgG preparation. The first process for the production of intravenous (*iv*) IgG was based on treatment of IgG with a quantity of pepsin (10) or plasmin (11) that caused enzymatic cleavage of IgG molecules. This process, corresponding to the preparation of first-generation *iv* IgG, is today considered to be obsolete (9).

Specific structural changes of IgG were introduced in the enzymatic methods to reduce anticomplementary activity. The products thus obtained usually have reduced *in vivo* survival times and their continuous use may cause an antigen response depending on the enzyme used (12). The second-generation *iv* IgG consisted of chemically modified preparations with more or less impaired Fc-related effector functions (9). From 1975 to 1989, the chemical modification of the protein was probably the most successful approach to the preparation of *iv* IgG (12). The reagents used for this modification range from ß-propiolactone (13) to reducing/alkylating agents (12,14), reducing/amidating agents (15) and reducing/sulfonating agents (16).

Today, all of these preparations are being replaced with third-generation products containing intact IgG molecules which retain effector functions. The latest generation includes preparations that are free of complement-activating aggregates thanks to the use of small or trace amounts of pepsin, pH 4.0, PEG and bulk adsorption with ion-exchange gel (9). This development was encouraged by the fact that the chromatographic method provides a safe and effective *iv* IgG product meeting the 1982 requirements of the World Health Organization Committee (17). The choice of a third-generation preparation which exhibits all the functions of the IgG molecule will be determined on the basis of safety in terms of viral transmission and absence of contaminants. Furthermore, the preparation should contain a normal distribution of IgG subclass molecules and have a half-life after infusion (9) which is in the physiological range of 21 to 36 days.

Material and Methods

Production equipment

Pharmacia liquid chromatography equipment was employed using a Bio Process controller (Uppsala, Sweden). The following columns were used: step 1, desalting, gel filtration on Sephadex G-25, 1 column, model BPSS 400/600 (60 cm in height by 40 cm in diameter). Step 2, anion-exchange DEAE-Sepharose FF, 1 column, model PS-370/15 (15 cm in height and 37 cm in diameter). Step 3, affinity gel Arginine Sepharose 4B (40%) + anion-exchange DEAE-Sepharose FF (60%), 1 column, model PS-370/15 (15 cm in height by 37 cm in diameter) and cation-exchange CM-Sepharose FF, 1 column, model Index 200/500 (15 cm in height and 20 cm in diameter). Step 4, cation-exchange CM-Sepharose FF, 1 column, model Index 200/500. Step 5, gel filtration on Sephacryl S-300, 1 column, model BPG 200/950 (95 cm in height and 20 cm in diameter).

Other instruments used were a tangential flow ultrafiltration Pellicon cassette system and a filtration system with a stainless steel sanitary filter holder, 293 mm in diameter (Millipore, Bedford, MA, USA), and a continuous flow centrifuge model AG BKA-6 (Westphalia Separator, Oeld, Germany).

Details about the buffer solutions are given in Table 1.

Preparation of immunoglobulin G

Approximately 1200 human plasma bags stored at -30°C were thawed at a temperature of 2 to 4°C in order to form a 200-l pool. Thawed plasma was centrifuged at 2°C to

obtain a cryoprecipitate to be used for the production of factor VIII. The supernatant of the cryoprecipitate was cleared by filtration through a 30-S depth filter (Zeta Plus, Cuno, Meriden, CT, USA) and desalted with coarse Sephadex G-25 filtration gel on a BPSS 400/600 column using 5 mM sodium acetate as elution solution. The pH was adjusted to 5.2 with 1 M CH_3CO_2H and the protein solution (410 l) was allowed to stand overnight in a cold chamber at 4ºC for euglobulin precipitation. On the following day the preparation was centrifuged at 4ºC to remove the precipitated euglobulins and the supernatant was cleared by filtration. The pH was readjusted to 5.2 with 1 M CH_3CO_2H and conductivity was adjusted to 1.4 mS/cm with NaCl. The sample was applied to a PS-370/15 column containing DEAE-Sepharose FF in order to separate gamma-globulin (unadsorbed) from other plasma proteins such as albumin, etc.

The gamma-globulin fraction was submitted to chromatography for the preparation of IgG as described below. The albumin fraction was further purified by a chromatographic method (18). The pH of the gamma-globulin fraction (480 l) was adjusted to pH 6.0 with 1 M NaOH and conductivity was adjusted to 1.4 mS/cm. The fraction was then applied to two columns, PS-370/15 and Index 200/500, coupled in series. The first column was packed with a mixture of two gels, 40% arginine Sepharose 4B and 60% DEAE-Sepharose FF, and the second with 8 l of CM-Sepharose FF gel.

Table 1 - Buffer solutions used.

Sephadex G-25 C	
Equilibration	5 mM sodium acetate, pH 7.0, and conductivity 0.37 mS/cm
Elution	with equilibration buffer
Cleaning and storage	0.5 M NaOH and 10 mM NaOH
DEAE-Sepharose FF	
Equilibration	20 mM sodium acetate, pH 5.2, and conductivity 1.4 mS/cm
1st Elution	with equilibration buffer
2nd Elution	25 mM sodium acetate, pH 4.5, and conductivity 1.75 mS/cm
3rd Elution	0.15 M sodium acetate, pH 4.0, and conductivity 8.0 mS/cm
Cleaning and storage	0.5 M NaOH and 10 mM NaOH
Arginine + DEAE-Sepharose and CM-Sepharose FF	
Equilibration	20 mM sodium acetate, pH 6.0, and conductivity 1.4 mS/cm
Elution of arginine + DEAE-Sepharose FF	0.5 M sodium acetate, pH 7.0, and conductivity >20 mS/cm
Elution of CM-Sepharose FF	1.0 M NaCl, pH 7.0
Cleaning and storage	0.5 M NaOH and 10 mM NaOH
CM-Sepharose FF (after virus inactivation)	
Equilibration	10 mM glycine, pH 7.0
Elution	0.1 M glycine + 0.15 M NaCl, pH 9.0
Cleaning and storage	0.5 M NaOH and 10 mM NaOH
Sephacryl S-300 HR	
1st Equilibration	0.1 M sodium acetate + 50 mM NaCl, pH 6.0
2nd Equilibration	0.15 M NaCl, pH 6.0
Elution	with 2nd equilibration buffer
Cleaning and storage	0.5 M NaOH and 10 mM NaOH

The proteins not of interest for the present study, IgM, IgA, transferrin, etc, were adsorbed to the first column. IgG was adsorbed to the second column and eluted and concentrated to 5% (w/v) using the Pellicon Cassette System 30,000 NMWL. The pH of the IgG solution (16 l) was adjusted to 5.5 and the material was submitted to viral inactivation with a solvent/detergent combination (19), i.e., 1% Tri (n-butyl) phosphate and 1% Triton X-100 at 35°C for 10 h. After viral inactivation, the solvent/detergent combination was removed by ion-exchange chromatography on CM-Sepharose FF in an Index 200/500 column containing 8 l of gel. The IgG solution was concentrated to 7%, pepsin was added (0.1 mg/g protein), pH 4.0, and the solution was heated at 37°C for 10 h. The preparation was cooled to 20°C and then applied to a BPG 200/950 column containing 20 l of Sephacryl S-300 HR filtration gel to remove the aggregated IgG molecules and pepsin. The eluted IgG solution was concentrated to 6.5% and then formulated as follows: the pH was adjusted to 5.0 with 1 N HCl, the conductivity to 9 to 10 mS/cm with solid NaCl, and 7.5% (w/v) maltose and 0.1 M glycine were added as stabilizers (12,20). The material was sterilized by filtration through a 0.22-µm membrane (Millipore) and the final product (14 l), containing 5% protein (w/v), was bottled in 50-ml type I (neutral) flasks, with 2.5 g IgG per flask and was stored at 4 to 8°C (see Figure 1).

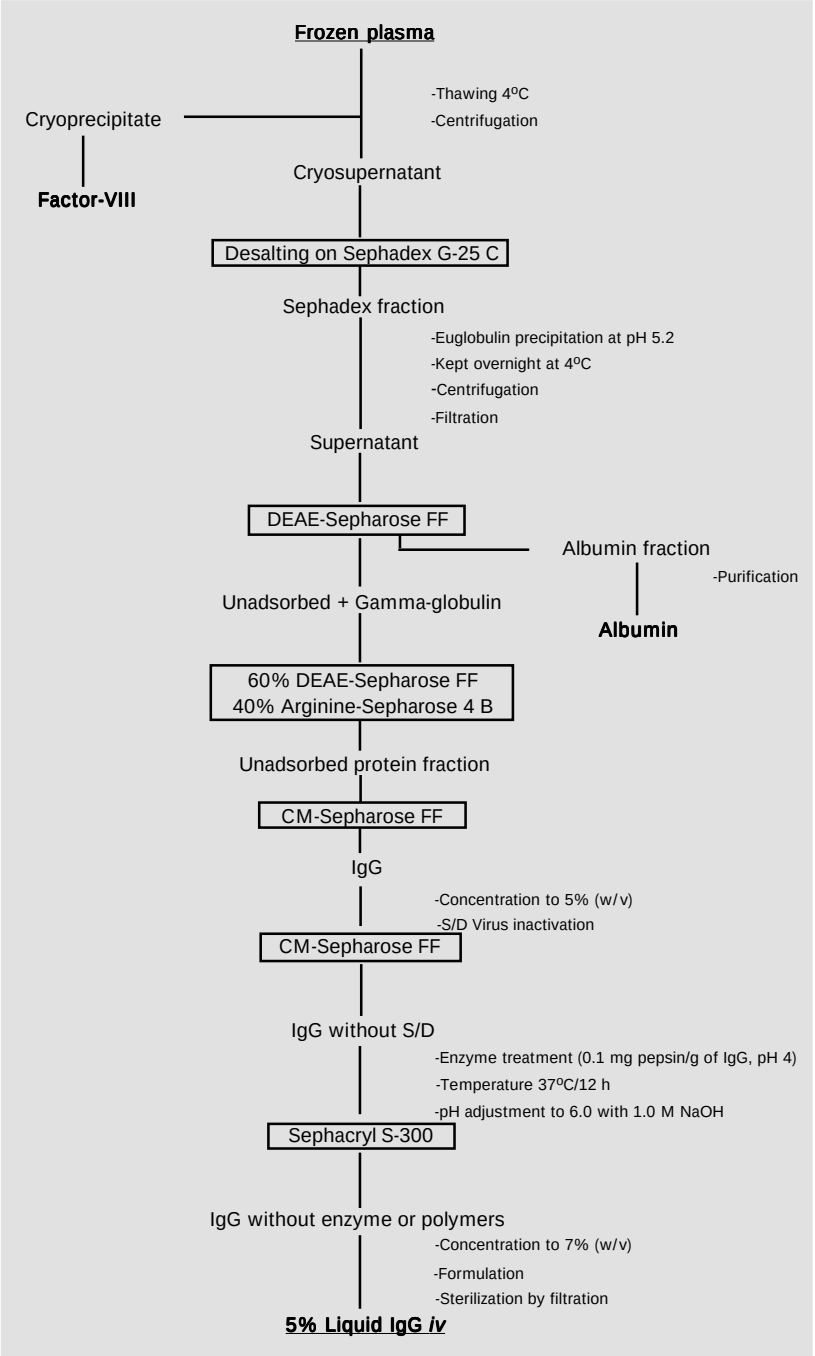

Figure 1 - Flow diagram for the production of liquid intravenous IgG.

Analytical methods

The characteristics of normal liquid 5% IgG for intravenous use were evaluated by the methods described in the Pharmacopée Européenne (1) and in Regulation No. 2.419 of 12/17/1996 of the Brazilian Ministry of Health.

The immunochemical tests were carried out by cellulose acetate electrophoresis, by the micro-Ouchterlony method and by immunoelectrophoresis using anti-total human protein and anti-animal protein antisera of domestic species such as horses, cows and sheep. Protein concentration was determined by the biuret method, pH was measured by

diluting to 1% in a 0.9% sodium chloride solution, and anti-A and anti-B hemagglutinins were determined by the indirect Coombs method. The presence of other plasma proteins such as IgA and IgM and of the IgG subclasses IgG1, IgG2, IgG3 and IgG4 was determined by radial immunodiffusion on Bindarid plates (The Binding Site Inc., San Diego, CA, USA).

Anticomplementary activity was determined by the method of Mayer using guinea pig complement and sheep red cells. The prekallikrein activator was determined using the S2302 chromogenic substrate of Chromogenix (Mölndal, Sweden).

The biological safety of the product was evaluated by tests for the detection of anti-HIV, anti-HTLV-1 and 2, anti-HCV, and HB antigens at the Serology Laboratory of Fundação Pró-Sangue Hemocentro de São Paulo. Sterility was evaluated by the Steritest membrane method (Sterility Testing System, Millipore). Pyrogenicity and toxicity were tested at Medlab, São Paulo, Brazil.

The distribution of monomers, dimers and polymers was evaluated by HPLC, and anti-polio I, II and III, anti-measles and anti-herpes activities were determined at Instituto Adolfo Lutz, São Paulo, Brazil. Anti-rubeola, anti-CMV and anti-streptolysin O activities were determined at the Immunology Laboratory of IAMSPE, São Paulo, Brazil. Stability was evaluated by incubating the preparation at 57°C for 4 h and observing the presence of jelling.

Results and Discussion

The size distribution of the product, evaluated by HPLC, indicated 94% monomers, 5.5% dimers and 0.5% polymers, corresponding to standard values. The profile of the IgG molecule did not show any alterations when the IgG preparation was incubated at 37°C for one month; the anticomplementary activity was 0.3 CH_{50}/mg IgG, in agreement with the specifications that determine a value of less than 1 CH_{50}/mg IgG, and the functional activity of Fc was unchanged, presenting an index of 125 to 128% of Fc, meeting the recommendation of Pharmacopée Européenne, which requires >60%. On the basis of these evaluations, we concluded that the product contained intact molecules. The subclasses determined were IgG1 = 65.6%, IgG2 = 28.7%, IgG3 = 4.2%, and IgG4 = 1.4%, with no significant variations compared to normal plasma. For prekallikrein activator (PKA) determination we used a reference PKA from the FDA and the S2302 chromogenic substrate of Chromogenix. Values of less than 5 IU/ml 5% (w/v) IgG were obtained, well below the specification limit of 35 IU/ml of PKA. The remaining data which characterize the product are presented in Tables 2 and 3, and cellulose acetate

Table 2 - Characteristics of liquid intravenous IgG.

N = 10. ND, Not detected. *Pharmacopée Européenne (1).

Analysis	Results	Specifications*
Protein concentration	5.0 ± 0.2%	>90 to <110%
pH determination	4.5-5.0	4.0-7.4
PKA determination	<5.0 IU/ml	<35 IU/ml
Purity (electrophoresis)	>99%	>95%
IgA	ND	–
IgM	ND	–
Anti-A hemagglutinin	≤1:8	<1:64
Anti-B hemagglutinin	≤1:8	<1:64
Anticomplementary activity	<0.3 CH_{50}/mg IgG	<1 CH_{50}/mg IgG
Aluminum	<20 µg/ml	–
Fc function	125-128%	>60%
Molecular distribution		
Monomers	94.0%	mono + dimers = 90%
Dimers	5.5%	
Polymers	0.5%	polymers <3%
IgG subclasses		
IgG1	65.6%	Subclass distribution
IgG2	28.7%	similar to plasma
IgG3	4.2%	
IgG4	1.4%	
Activities		
Anti-polio	+	present
Anti-rubeola	+	present
Anti-CMV	+	present
Anti-streptolysin O	+	present
Pyrogens	satisfactory	satisfactory
Abnormal toxicity	satisfactory	satisfactory
Stability (heating to 57°C/4 h)	no jelling	no jelling

electrophoresis and immunoelectrophoresis data are shown in Figures 2 and 3, respectively.

We describe the preparation of intravenous 5% immunoglobulins in the liquid state from a pool of human plasma from 1200 donors by the chromatographic method. The analyses performed for quality control showed that the IgG met international specifications. In the stability test involving heating to 57°C for 4 h there was no jelling, and in the quarantine test involving incubation in an oven at 37°C for 4 weeks, again there were no alterations in the IgG molecule.

Viral inactivation was performed with a solvent/detergent system (19), i.e., 1% Tri (n-butyl) phosphate and 1% Triton X-100, pH 5.5, at 35°C for 10 h, which was then removed by the ion-exchange gel, CM-Sepharose FF. The protein aggregates generated during the process were removed by gel filtration chromatography through Sephacryl S-300 HR. Thus, the product obtained consisted mainly of the monomer, presenting only trace amounts of polymers, consistent with low anticomplementary activity, i.e., less than 1 CH_{50}/mg IgG.

Of the several stabilizing agents tested, glucose, sucrose and maltose, the last one at a 7.5% concentration, and 0.1 M glycine, pH 5.0 (12,20), were found to be the most appropriate. The IgG solution was limpid and transparent with no detectable molecular alterations even when stored for more than 2 years at 5 to 8°C.

On the basis of *in vitro* and *in vivo* laboratory tests, we conclude that the product fully satisfies all the requirements of the Pharmacopée Européenne (1), as well as the norms of the Brazilian Ministry of Health (Regulation No. 2419 of December 17, 1996). We are awaiting the results of clinical tests currently underway to liberate the product for use.

Table 3 - Antibodies detected in liquid intravenous IgG.

N = 10.

Antibody	Method	Titer and/or unit
Anti-polio I	Neutralization test	1:256
Anti-polio II	Neutralization test	1:128
Anti-polio III	Neutralization test	1:64
Anti-measles	Hemagglutination inhibition	1:64
Anti-rubeola	ELISA	756 IU/ml
Anti-herpes	Immunofluorescence	1:128
Anti-cytomegalovirus	ELISA	898 IU/ml
Anti-streptolysin O	Nephelometry	716 IU/ml

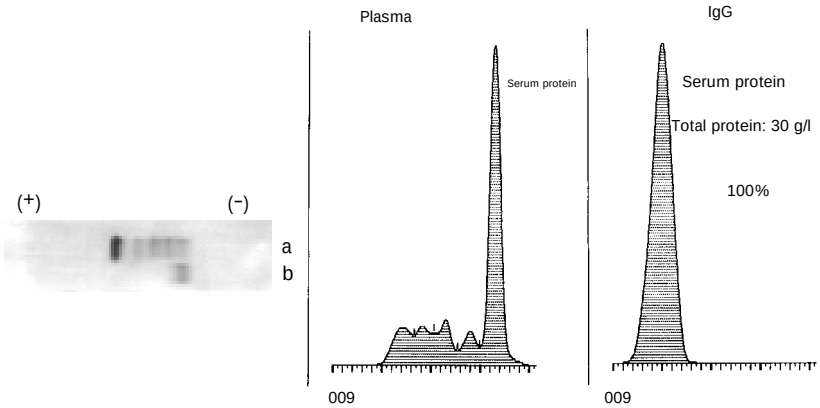

Figure 2 - Cellulose acetate electrophoresis. *a*, Plasma pool (25 µl of 10 g/l); *b*, immunoglobulin G (25 µl of 30 g/l). Protein was detected with Ponceau S and the data are reported as percent of total densitometer units.

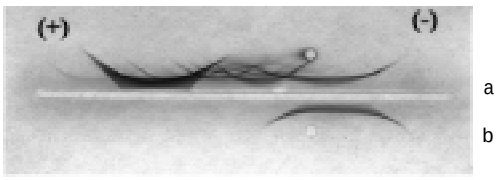

Figure 3 - Immunoelectrophoresis. *a*, Plasma pool (2 µl of 10 g/l); *b*, immunoglobulin G (2 µl of 30 g/l). Protein was detected with light green stain.

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