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Original Article

Quality Evaluation and Comparison of Immunoglobulin Prepared by Two Various Methods of Human Plasma Fractionation

Mehdi Bagheri[a] , Sima Mohammadi Bidhendi[a], Hashem Khorsand Mohammad Pour[b], Soudabeh Banazadeh[c], Ali Akbar Pourfatolah[b], Parviz Kokhaei[d] ,Seyed Mehrdad Jalali[e], Afsaneh Aghaie[b*]

[a]Immunology Department, Semnan University of Medical Sciences, Semnan, Iran, [b]Blood Transfusion Research center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran, [c]Pishro Tashkhis Fardavar Company, Tehran, Iran, [d]IGT Lab. CCK, R8, Karolinska University Hospital, Solna, 171-76 Stockholm, Sweden, [e]Iranian Blood Research and Fractionation Company, 1449613111, Tehran, Iran.

Abstract

Based on European Pharmacopeia, there are some features which should be measured for any intravenous immunoglobulins prior to final release of the product. The most critical ones are the level of prekallikrein and anti-complementary activity in final formulation. For all commercial products, the national reference laboratory is prone to conduct such tests and there is no local report on quality control tests done on the products derived from Iranian human plasma. The study is to measure and control the international requirements such as prekallikrein count and anti-complementary activity for human intravenous immunoglobulins manufactured by local developed process in Iran in pilot scale. IgG-rich fraction was obtained by two fractionation methods. Cryoprecipitate was separated from tested fresh frozen plasma in both methods. In method I, for the next steps, fraction I paste, fraction II+III paste, and at the end, the fraction II paste were precipitated. In method II, the fraction I+II+III paste was simultaneously precipitated followed by deriving the fraction II paste. The paste obtained by both methods was separately subjected to the purification processes using anion and cation exchange chromatography followed by gel filtration and activity level of Prekallikrein in addition to anti-complement activity were compared with other laboratory evaluations. No difference was illustrated between protein and albumin content, pH, and conductivity of the two products. The fraction II paste obtained from both methods was measured and compared with each other. The IgG yield compared to the primary plasma was calculated as 4.6 and 4.3 g for the aforementioned methods respectively. The absence of impurities was determined by a strong IgG bond in electrophoresis while by HPLC, the dimer/ monomer content was measured more than 99% and the polymer/ aggregate was less than 1%. The amount of prekallikrein and total anti-coagulant activity met the European Pharmacopoeia requirements for both methods.

Keywords: Activator, Anti-Complement Activity, Cohn's method, Fraction II paste, Plasma Fractionation, Prekallikrein, Separation Technology.

Corresponding Authors: Afsaneh Aghaie, Blood Transfusion Research center, High Institute for Research and Education in Transfusion Medicine, Tehran, Iran
Tel: +21 88629608
Email: aghaie.a@gmail.com
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1. Introduction

Immunoglobulin G (IgG) isolation process from human plasma by cold ethanol fractionation was primarily performed in the 1940s by Cohen. This method allowed the production of albumin and IgG based on the physicochemical parameters such as ethanol concentration, pH, ionic strength, and protein content [1].

Initially, human intra muscular immunoglobulin G therapy was the only strategic therapy to replace antibodies in immunodeficiency diseases [2] and due to adverse side effects subjected to the changes in the IgG molecule, such as the accumulation resulted by ethanol fractionation process and other factors increasing Anti Complements Activation (ACA), could not be injected intravenously [3]. Formulation of intravenous immunoglobulin (IVIG) was developed in the late 1970s and was largely a substitute for muscular cure and very effective in preventing infection [4-6].

Over the past few decades, IVIG has become an important treatment in clinical medicine. The main use of these medicaments has been antibody replacement. However, in many other clinical cases, IVIG treatment has also been shown to be effective [7].

Most of the IVIG products in the world are still being produced by cold ethanol fractionation [8]. Performing and combining ion exchange, affinity and size exclusion chromatography significantly improved the quality of immunoglobulin products [9].

On the other hand, Prekallikrein Activator (PKA) is a contamination in IVIG produced from plasma fractionation [10]. High levels of PKA cause adverse vascular complications in patients. Also, PKA initiates a cascade of enzymatic reactions and activates prekallikrein in conversion to kallikrein. The high molecular weight Kininogen is then segregated by kallikrein and bradykinin is released, which causes dilatation and increased vascular permeability which leads to hypotension in the patient [11]. For this reason, the measurement of PKA in immunoglobulin products is one of the most essential requirements in the European Pharmacopoeia [12].

In this study, two different plasma fractionation processes were carried out to obtain IgG-rich fraction II paste. In the first method, the cryo-rich plasma was precipitated followed by fraction I paste, fraction II+III paste, and finally, the fraction II paste precipitation at different concentrations of ethanol, pH and ionic strength. Via the second method, having the cryo precipitated, the fraction I+II+III paste was moved out in one step simultaneously to obtain the fraction II paste. Purification processes were subsequently performed by anion exchange chromatography, cation exchange

chromatography and gel filtration on both types of pastes II separately and the resultant immunoglobulin product was evaluated by laboratory tests. To achieve the goal of this study, both the prekallikrein activator test and anti-complement activity measurements were primarily conducted and then the immunoglobulin product obtained from the two fractionation methods was compared in this regard.

2. Material and Methods

2.1. Plasma Supply and Preparation for Fractionation

In order to remove the cryo-rich plasma in each manufacturing series on a pilot scale, 80 FFP bags were selected from the -30 ° C freezer and transferred overnight to the -4 ° C refrigerator. Plasma units were disinfected by floating in a vessel half loaded with alcohol 70% for 1 h. Then they were cut and 14 liter of plasma was transferred to a double-glazed stainless steel tank (SEN, Germany). Having been melted, plasma was transferred to a continuous centrifuge (Cepa Z41, Germany) at about 15,000 g at 4 ° C where cryo paste for factor VIII was precipitated and removed.

2.2. Plasma Fractionation Method 1

2.2.1. Fraction I Paste Precipitation

Cryo Poor Plasma (CPP) was transferred to the fractionation tank and its pH was increased to 7.5, then ethanol was added at a concentration of 9%. Being stored for 2-3 h at a temperature of + 3 ± 1 ° C and to complete the fractionation, it was centrifuged at a temperature of -2.5 ± 1 ° C at 15000 g resulted in precipitation of fraction I paste containing fibrinogen and the supernatant I was then collected for the rest of the process.

2.2.2. Fraction II+III Paste Precipitation

The supernatant I was transferred to the fractionation tank and the pH was adjusted to 5.9 and ethanol was added at a concentration of 20%. Having been kept agitating for 4 h at -6 ± 1 ˚C and in order for the precipitation to be completed, it was centrifuged at -6 ± 1 ° C within 15000 g led to the fraction II +III paste precipitation.

2.3. Plasma Fractionation Method II

2.3.1. Fraction I+II+III Paste Precipitation

CPP was transferred to the fractionation tank and the pH was adjusted to 5.9 and ethanol was added at a concentration of 20%. Having been kept for 2 h at a temperature of 7 ± 1° C and to complete the precipitation, it was centrifuged at a temperature of -7 ± 1° C within 15000 g and the fraction I+II+III paste was simultaneously precipitated in one step.

2.3.2. Paste Dissolution

700 g of the fraction II + III paste was weighed and added up with distilled water in 12 times the weight of the paste, then it was poured into the fractionation tank. After the paste was dissolved, the pH was adjusted to 4.8 and made homogeneous for 2 h in the fractionation tank. Then the pH was adjusted to 5.1 and having rotated for 2 h in the tank and ethanol was injected at a concentration of 14%. Having been kept rotating for 2 h at a

temperature of -3 ± 1 ° C, it was centrifuged at 6 ± 1 ° C within 15000 g where the fraction III paste was removed and discarded. The fraction III supernatant was passed through the Sartorius filtration system and prefilters via a peristaltic pump and then transferred to the fractionation tank. In the case of I+II+III paste, 700 g of paste weighed and all the above steps were repeated and consequently, the fraction III supernatant was transferred to a fractionation tank during a separate process.

2.4. Fraction II Paste Preparation

The next fractionation steps on the fraction III supernatant obtained from the two different fractionation methods were similarly continued but in separate. Fraction III Supernatant from both methods was transferred to the fractionation tank and 4.5 grams of NaCl per liter was added. Ethanol was then injected at a concentration of 25% following to the pH adjustment to 7.2 ± 0.1 and it was agitated for 2 h to be homogeneous and subsequently, centrifuged at a temperature of 8 ± 1°C within 15000 g. Both types of fraction II precipitate (Paste II) containing IgG were quickly collected, weighed and transferred to the -70 °C Freezer.

2.5. Purification

Both types of paste II derived were purified by the same processes of chromatography. Anion exchange chromatography was used to remove negatively charged proteins. Then, to reach greater purity of IgG, a CM Sepharose FF cation exchange chromatography was carried out and exited elute was transferred to gel filtration column to be more purified. Before these, the paste II was dissoluted by 8-10 times deionized water up to the concentration of 4g/100g protein and then went through a filter system of 0.45 micron (Whatman, USA).

2.5.1. Anion Exchange Chromatography

Pharmacia chromatography column was chosen at a height of 20 cm and a diameter of 16 mm and 30 ml of Q Sepharose FF gel (Pharmacia, Sweden) was gradually poured into the column and was equilibrated by 0.02 M sodium acetate buffer at pH 6. The filtered suspension was then loaded on the column with a pH of 6. IgG did not connect to the gel due to its positive electrical charge it exited from the column along with the elution buffer. Then it was collected at 4 ° C and the column was retrieved.

2.5.2. Cation Exchange Chromatography

Pharmacia chromatography column was selected at a height of 20 cm and a diameter of 16 mm and 30 ml of the CM Sepharose FF gel (Pharmacia, Sweden) was gradually poured into the column and was equilibrated by a 0.02 M sodium acetate buffer with a pH of 5.4. The elution obtained from former step was loaded to the column with a pH of 5.4. Passing the 0.02M sodium acetate wash buffer through the column, the undesired proteins were removed and the IgG was attached to the column due to its positive charge. Then, sodium chloride 0.2 M in acetate buffer (as elution buffer) passed through the column, ultra violet peak related to

IgG was collected at 4 ° C and the column was retrieved.

2.5.3. Gel Filtration

Pharmacia chromatography column was selected at a height of 70 cm and a diameter of 16 mm and 60 ml of Sephacryl S-300 gel (Pharmacia, Sweden) was gradually poured into column and the column was equilibrated by 0.9% sodium chloride. The IgG suspension taken from the cation exchange chromatography, was concentrated by an ultrafiltration system using Viva-Spine tubes (Sartorius) about 5 times up to the concentration of 5 g/100g. The filtered suspension was loaded onto the column. Elution buffer was then passed through the column, the IgG peak was collected at 4 ° C and the column was retrieved.

2.6. Concentration via Ultra/Diafilteration

It was needed to boost the IgG concentration from the Q Sepharose FF which was 0.7g/100g. Therefore, an ultrafiltration step was performed using a 0.3M glycine buffer at pH 5 to reach a concentration of 5g/100g. Then, the product was diafiltered against ultrafiltration buffer (sodium acetate 0.02 M, glycine 0.3 M, pH 5, and conductivity of 1.4 ms/cm).

2.7. Laboratory Tests

2.7.1. SDS-PAGE

In order to investigate the purity of different samples resulted from fractionation process, SDS-PAGE was conducted on 5% stacking gel and 12% resolving gel in absence of reductive substances (Pharmacia). Dissoluted paste II obtained from both fractionation methods (paste and final product) and a commercial IgG product (as control sample) were loaded in each well and the bands were stained with Coomassie Brilliant Blue R250 (Merck). Cellulose acetate electrophoresis was performed on the final products as well.

2.7.2. HPLC

To investigate the polymerization and accumulation, an HPLC test was performed by the HPLC system (Waters, USA) and the TSK-G3000 SWXL column 300 × 6mm (Tosoh Bioscience, Japan). Solutions were filtered (Millipore, USA) and degassed by a vacuum pump. Samples were injected to the system after 0.45 nm Millipore filtration.

2.7.3. Determination of Protein and Albumin Content

The protein content of both products were measured by Biuret method (546 nm) and Albumin content was determined by bromocresol green method (630 nm).

2.7.4. Determination of PKA Content

In order to measure the amount of prekallikrein activator (PKA), prekallikrein (PK) was primarily purified from human plasma and then, PKA levels were determined in different products.

2.7.4.1. PK Preparation

Human plasma was isolated from a volume of 50 ml of citrated blood and adjacent to the

Tris buffer of 0.05 M with a pH 8 transferred to a dialysis bag (Sigma, USA) to be rotary dialyzed for 24 h at room temperature in addition to a buffer replacement in every 6 h. The dialyzed plasma was then gradually poured into a polymeric plastic column of 2.5 * 30 cm filled with DE-52 gel. Without activation, this is for the plasma prekallikrein not be bonded to the column so it will remove from the column with an amount of IgG. The absorbance was monitored at 280 nm and absorbent tubes containing prekallikrein were collected. Prekallikrein specimens were stored at -25 ° C following to the addition of sodium chloride up to 0.75 g/100g and 0.45 nm filtration (Millipore, USA).

2.7.4.2. Measurement of PK Amount

This test was based on an enzyme assay and all stages were carried out in plastic tubes to prevent undesired PKA activation in contact with the glass. The PKA assay consisted of two reaction steps. In the first step, PKA was incubated with prekallikrein to be converted to kallikrein. In the second step, kallikrein, a serine protease, catalyzed the separation of paranitroaniline from the chromogen substrate (S-2302). The amount of released paranitroaniline, which was proportional to the amount of kallikrein and ultimately proportional to the amount of PKA in the solution at constant temperature, was measured by spectrophotometry at 405 nm in comparison with the various concentrations of the WHO International Standard.

2.7.5. Anti-Compliment Activity (ACA)

To measure ACA, an antibody-mediated sheep red blood cell lysis (RBC) method was established as a reference method by the European Pharmacopoeia in 1995 [13]. Sheep red blood cells (SRBC), hemolysin and complement were purchased from Razi Institute of Iran. Using SRBC of 5%, the appropriate dilution of hemolysin to make the sheep red blood cells sensitized, tittered, the supernatant absorption was determined at 541 nm and the hemolysis percentage was calculated. The best hemolysin dilution is the one in which there is no increase in the hemolysis while increasing the hemolysin concentration. This dilution is called MHU (Minimal Hemolytic Unit). To provide optimal SRBC sensitization, an appropriate volume of diluted hemolysin containing 2 MHU/ml (a volume equivalent to 5% SRBC) was prepared. Utilizing appropriate gelatin barbiturate, concentrations of the complement were prepared and the complement titration was performed using sensitized sheep red blood cells to obtain the appropriate titer for hemolysis of the sheep red blood cell. The CH50 was calculated and the complement solution of 100 CH50/ml was prepared by gelatin barbiturate. 10 mg of IgG product obtained from both methods incubated with 20 CH50/ml complement and the remaining complement activity was calculated. The levels of transferrin, alpha-1 anti-trypsin, immunoglobulin G, immunoglobulin A, immunoglobulin M and IgG subclasses was measured and compared by nephlometric method in the first method samples (including

CPP, supernatant I, supernatant II + III, supernatant III, and the final product) and the second method samples (including CPP, supernatant I, supernatant I + II + III, supernatant III and final product).

3. Results and Discussion

The results for pH, conductivity and temperature in different fractions of fraction II + III, fraction I+II+III and fraction II paste, as well as the results for cellulose acetate electrophoresis and protein and albumin content were summarized in Table 1, Table 2,

Table 1. pH, conductivity and temperature results for various fractions during fractionation of II+III and I+II+III fractions.

	pH	**Conductivity (μS/cm)**	**Temperature (°C)**
FFP (Fresh Frozen Plasma)	7.74	12	4
Cryosupenatant	7.9	11.45	4
Supernatant I (Method I)	5.98	8	-3
Supernatant II+III (Method I)	6.03	1.9	-5
Fraction II+III paste (Method I)	6.7	1	4
Supernatant III (Method I)	5.2	0.5	-5
Supernatant II (Method I)	7.2	0.8	-5
Fraction II paste (Method I)	7.6	1	4
Supernatant I+II+III (Method II)	6.28	4.63	-6
Supernatant II (Method II)	7.25	0.8	-5
Fraction II paste (Method II)	7.6	1	4

Table 2. Cellulose acetate electrophoresis results for fraction II paste.

	Cellulose acetate electrophoresis (%)				
	Alb	α1	α2	β	γ
Plasma (FFP)	57.7	2.1	8	11	21
FII Method I	0.1	0	0	0	>99
FII Method II	0.1	0	0	0	>99

Table 3. Protein and albumin content in fraction II paste.

	Protein (g/100g)	Albumin (g/100g)
Plasma (FFP)	6.8	4.3
FII paste Method I	4.8	0.37
FII paste Method II	5	0.37

and 3 respectively.

The results for the SDS-PAGE of various fractions are shown in Fig. 1, which showed only a strong bond in the IgG molecular weight region.

Fraction II paste from two methods was

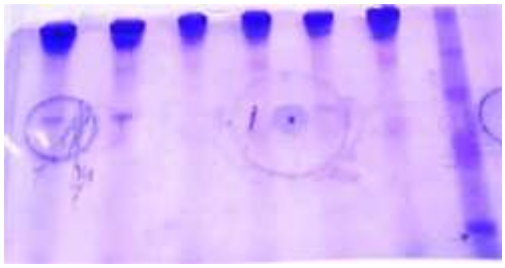
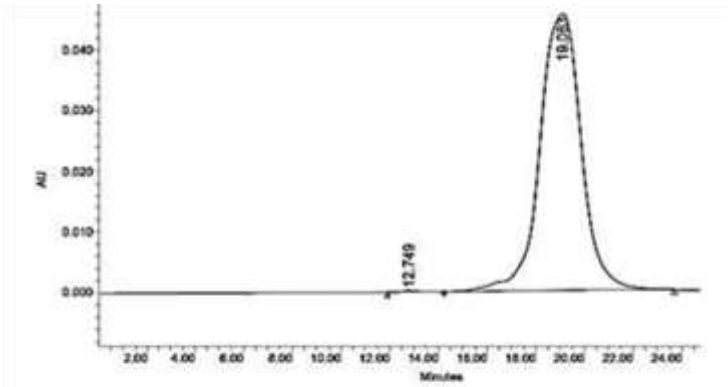
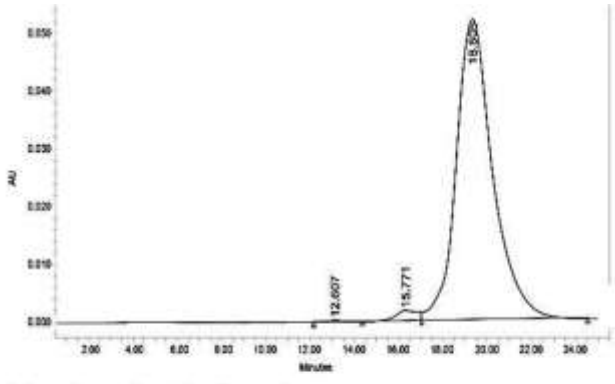


Figure 1. Cellulose acetate electrophoresis results. Right to left: Marker (HMW), Commercial IVIG, IgG pick (after cation exchange chromatography), FII method II (after gel filtration), FII method 1 (after gel filtration), FII method II (before chromatography with extra bonds), FII method I (before chromatography with extra bonds).



	RT (min)	Area (V*sec)	% Area	Height (V)	% Height
1	12.749	14906	0.27	310	0.68
2	19.053	5425687	99.73	45607	99.32

Figure 2. HPLC results for method I fractionation.



	RT (min)	Area (V*sec)	% Area	Height (V)	% Height
1	12.607	11752	0.19	324	0.60
2	15.771	134020	2.18	1885	3.47
3	18.805	5995234	97.63	52105	95.93

Figure 3. HPLC results for method II fractionation.

tested by HPLC and the results are presented in Fig. 2 and Fig.3 in terms of molecular weight distribution, polymer / aggregate and dimer / monomer.

The yield in different methods of fractionation was calculated by two different methods and is shown in Table 4.

The PKA amount was measured less than 20 IU / ml on the basis of absorption of the standards, the final product obtained from two methods of fractionation and commercial control samples.

The absorbance of different fractions from two methods of fractionation and commercial

Table 4. Yields of products derived via different methods of fractionation.

Yield / Sample	Method I (gr/kg plasma)	Method II (gr/kg plasma)
Plasma	14L	14.5L
CPP	9.64	10.1
Fraction I paste	10.8	0
Fraction II+III paste	58.5	0
Fraction I+II+III paste	0	66.2
Fraction III paste	35	36.5
Fraction I paste	10.14	10.34

Table 5. Nephlometric results for derived fractions.

	Transferrin g/L	ASO	α1- Antitrypsin	IgG	IgM	IgA
Plasma	3.025	63.780	0.995	592	115	164
Method I						
CPP	1.628 1.999	47.480	0.962	635	126	148
Supernatant I	2.248	27.900	0.569	1411	68	122
Supernatant II+III	1.851	<16.680	0.389	44	<3 ND	5
Supernatant III	<0.407	21.350	<0.364	859	<3 ND	ND
Final	<0.407	168.700	<0.364	2333×2 >3500	<3 ND	5
Method II						
CPP	2.365	64.980	0.783	1600	86	152
Supernatant I+II+III	1.489	<16.680	<0.364	61	<3 ND	6
Supernatant III	<0.407	<16.680	<0.364	917	<3 ND	ND
Final	<0.407	206.900	<0.364	2437×2 >3500	<3 ND	4
Low Control	2.012	83.390	0.485 0.874			0.93- 1.39
High Control	5.409	363.700	1.307			2.62- 3.94
Normal Range	2.24-4.06 g/L	27-604 IU/mL	0-629-1.696 g/L			0.86- 3.20

control samples was read at 541 and according to the following formula, ACA in two fraction II pastes obtained from two fractionation methods was calculated as of ≤ 1 CH50/mg IgG.

$$\left[\frac{\left[complement\ \left(\frac{CH50}{ml}\right) - sample\ \left(\frac{CH50}{ml}\right)\right]}{protein\ \left(\frac{mgr}{ml}\right)}\right] = ACA\ \left(\frac{CH50}{mg}\right)$$

Nephlometric results showed no significant difference in the amount of transferrin, alpha-1 anti-trypsin, immunoglobulin G, immunoglobulin A, immunoglobulin M and IgG subclasses in the fractions and the final product of both methods. The results are summarized in Tables 5 and 6.

Intravenous Immunoglobulin G (IVIG) is currently one of the most widely used plasma components in the world, and its annual consumption has grown to hundreds [13]. After years of Cohn's plasma fractionation, the production of immunoglobulins is still convinced on cold ethanol-based fractionation. However, over the years, improved purity, increased productivity and reduced production costs have been the main concerns of manufacturers [14].

In 2000, Tanaka and colleagues produced IVIG from Cohn's fraction II + III paste by chromatography. In this method, an anion exchange and then a cationic and finally size exclusion chromatography were used [15]. Lebling et al. also used caprilate in the purification process together with chromatography [16].

These methods do not seem to be cost-effective due to the high cost of process. In 2001, Sisti and his colleagues prepared the fraction II paste via several stages of filtration, as well as diafiltration of the IVIG product in two liquid lyophilized formulations [17].

Although it is thought that the use of immunoglobulin is relatively safe for intravenous use, a number of side effects have been attributed to the use of IVIG. There are many causes for these complications,

Table 6. Nephlometric results for IgG subclasses.

	IgG1 mg/L	IgG2 mg/L	IgG3 mg/L	IgG4 mg/L
Plasma	4518	3418	442.500	634.900
Method I				
CPP	3598	2807	371.600	540.100
Supernatant I	3073	3135	284	456.100
Supernatant II+III	<1449	<808.900	<79.750	<53.480
Supernatant III	<1449	1582	95.090	ND
Final I	12400	996.200	725.200	738.400
Method II				
CPP	4027	ND	ND	ND
Supernatant I+II+II	<1449	<808.900	<79.750	<53.480
Supernatant III	ND	1207	ND	ND
Final II	ND	ND	835.900	>848
Low Control	2567	1806	150.800	155.100
High Control	6670	6872	486.800	477.800
Normal Range	3730-9610	2220-5740	286-1330	62-1127

including contamination with PKA and kallikrein [18]. During the plasma fractionation, Cohn's method produces vasoactive materials such as PKA. The high levels of PKA in products made from human plasma fractionation using the Cohn's method are considered as contamination and cause adverse vascular responses [10]. For this reason, one of the tests required by the European Pharmacopoeia to produce IVIG from human plasma is to measure the amount of PKA in the manufactured product. Therefore, it is very important to create a simple and repeatable method for determining the amount of PKA in injectable products.

IgG aggregates in IVIG are also always considered as one of the main causes of side effects. The presence of IgG in IVIG is associated with the activation and use of complement without antigen involvement [19]. The formation of immune complexation resulting in aggregation activates complement and releases of anafilatoxins such as C3a, C4a and C5a in the bloodstream [18]. Therefore, the measurement of anti-complementary activity in the immunoglobulin product is considered as a crucial aspect in the evaluation of drug quality control [12].

Different methods have been used to reduce these complications. We tried to achieve the lowest possible use of chemicals during the process. In addition, due to neutral isoelectric point of immunoglobulin G, acidic pH and low ionic concentration were used to sediment proteins other than immunoglobulin G. The use of acidic pH for the fractionation of fraction II+III was also reported in another study by Lebling et al. in the production of IVIG by chromatography and caprilate [16].

Over the years, various manufacturers have used a variety of methods to improve the production and removal of polymers. At present, with the integration of the industrial process of plasma fractionation and chromatographic methods, it is possible to isolate the biological compounds with adequate purity, to keep the biological characteristics untouched in the industrial scale and to improve the yield of IgG with a higher degree of purity with fewer side effects as well. Thus, with the development of chromatographic methods, the quality of immunoglobulins dramatically increased [19].

In this project, with the modification of the Cohn's method, IgG-rich II paste was obtained by two different fractionation methods, and the Fraction II paste obtained from two different processes was dispensed under an anion exchange chromatography using the FF gel QF and cation exchange chromatography using The CM gel and finally gel filtration using the Sephacryl S-300 gel was used as the final step for polishing the product and in order to eliminate protein contamination from the plasma mixture. Finally, the yield, purity and biological parameters in the paste II obtained from the two methods were compared.

In 2000, Tanaka and colleagues also produced IVIG from Cohn's fraction II + III paste by chromatography [15]. Receiving IgG at 4.6 and 4.3 grams per liter of plasma in the first and second methods showed good yields compared to the method used by Tanaka and colleagues at 4.3 grams. IgG recovery rates

were 39% and 40%, respectively, in both methods. pH, conductivity as well as protein and albumin content in different fractions illustrated that the paste II did not show different results in two methods.

The results of cellulose acetate electrophoresis in the final products obtained from the two methods and the control samples, demonstrated a purity higher than 99%. IgG, and IgM levels in both pastes were less than 0.5% and IgA levels were either less than 1.2%. SDS-PAGE electrophoresis using Coomassie Blue in absence of reducing materials on paste II produced by two methods, showed only a strong band in the IgG molecular weight region. It was demonstrated that European Pharmacopeia requirements could be admissibly provided by fraction II paste derived from two various plasma fractionation methods, specifically in comparison with the IVIG commercial sample, in terms of the amount of prekallikrein activator, anti-complement activity, dimer / monomer and polymer / aggregates.

4. Conclusion

The aim of this study was to find a short and affordable method to prepare immunoglobulin and quantitative and qualitative comparisons of the product obtained by two different methods of fractionation by laboratory tests. In general, one of the goals in changing the processes of plasma fractionation is to reach a cost beneficial route of large scale production. So eliminating one step of the process will not only save time but also reduce the consumption of substances, buffers, and ethanol, which can decline the ethanol content by about 10% and shorten the time and process steps. The experiments showed that in addition to shortening the process compared to the current methods, the proposed method has the necessary capabilities to produce cost-effective, to keep the quality and safety of the product, and via complementary tests, it will provide the manufacturers with the necessary measures in order to implement this protocol in large industrial scale.

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