US 20070049732A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2007/0049732 A1**

Zurlo et al. (43) **Pub. Date: Mar. 1, 2007**

(54) **ULTRA-HIGH YIELD INTRAVENOUS IMMUNE GLOBULIN PREPARATION**

(76) Inventors: **Eugene J. Zurlo**, Kiawah Island, SC (US); **Dennis Curtin**, Peru, NY (US); **Allan L. Louderback**, Temple City, CA (US)

Correspondence Address:
EUGENE J. ZURLO
4 Nicklaus Lane
Kiawah Island, SC 29455 (US)

(21) Appl. No.: **11/217,956**

(22) Filed: **Sep. 1, 2005**

Publication Classification

(51) **Int. Cl.**
C07K 16/18 (2007.01)
C07K 14/765 (2007.01)

(52) **U.S. Cl.** **530/387.1**; 530/383

(57) **ABSTRACT**

An efficacious large-scale alcohol-free plasma fractionation production process which produces a high-yielding, non-denatured, double viral-inactivated intravenous human immune gamma globulin (IgG) product. The process employs sodium citrate in two initial fractionation steps, followed by diafiltration to remove sodium citrate.

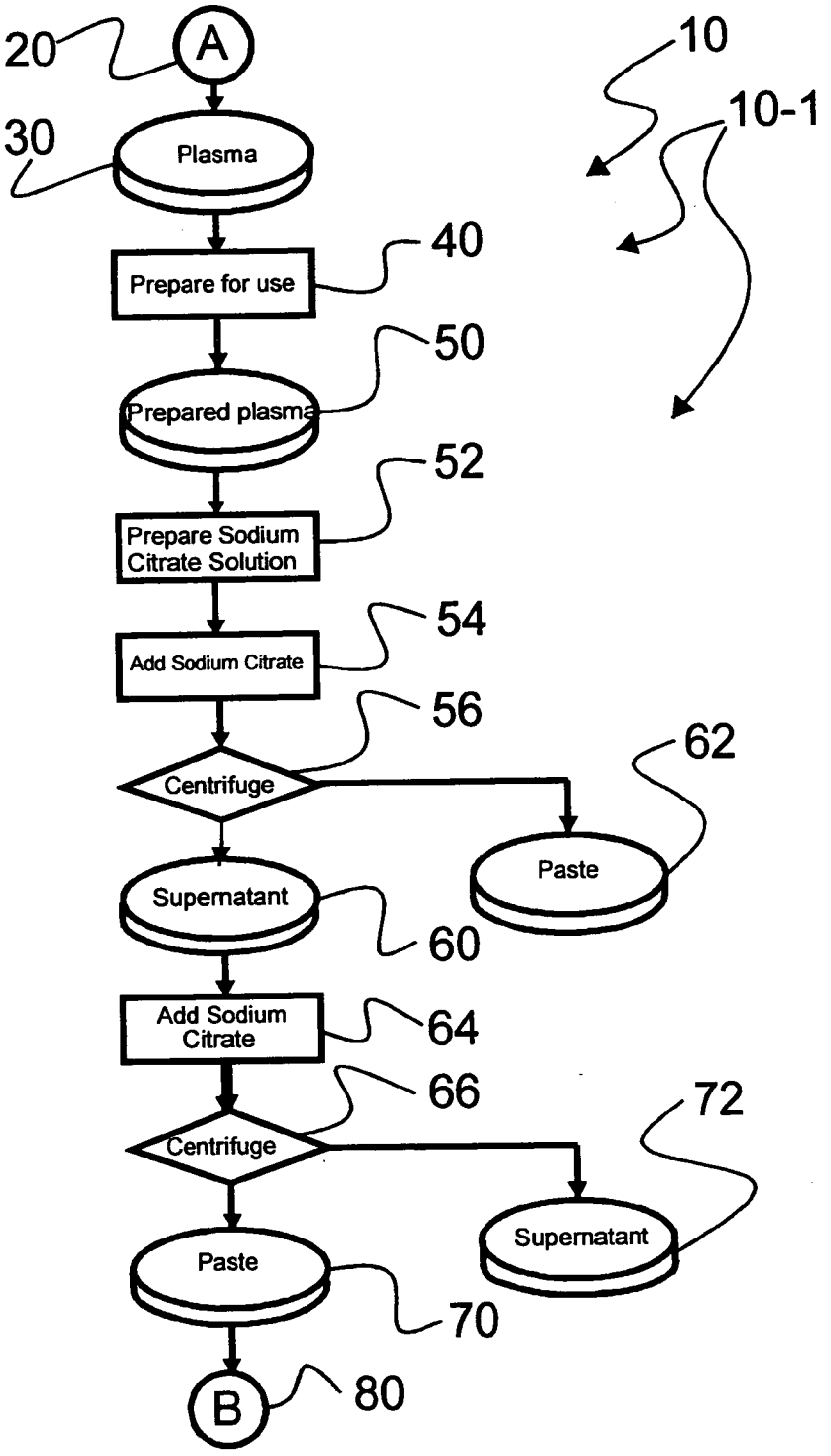
20
A
30
Plasma
10
10-1
40
Prepare for use
50
Prepared plasma
52
Prepare Sodium Citrate Solution
54
Add Sodium Citrate
56
Centrifuge
62
Paste
Supernatant
60
Add Sodium Citrate
64
66
Centrifuge
72
Supernatant
Paste
70
B
80

Figure 1

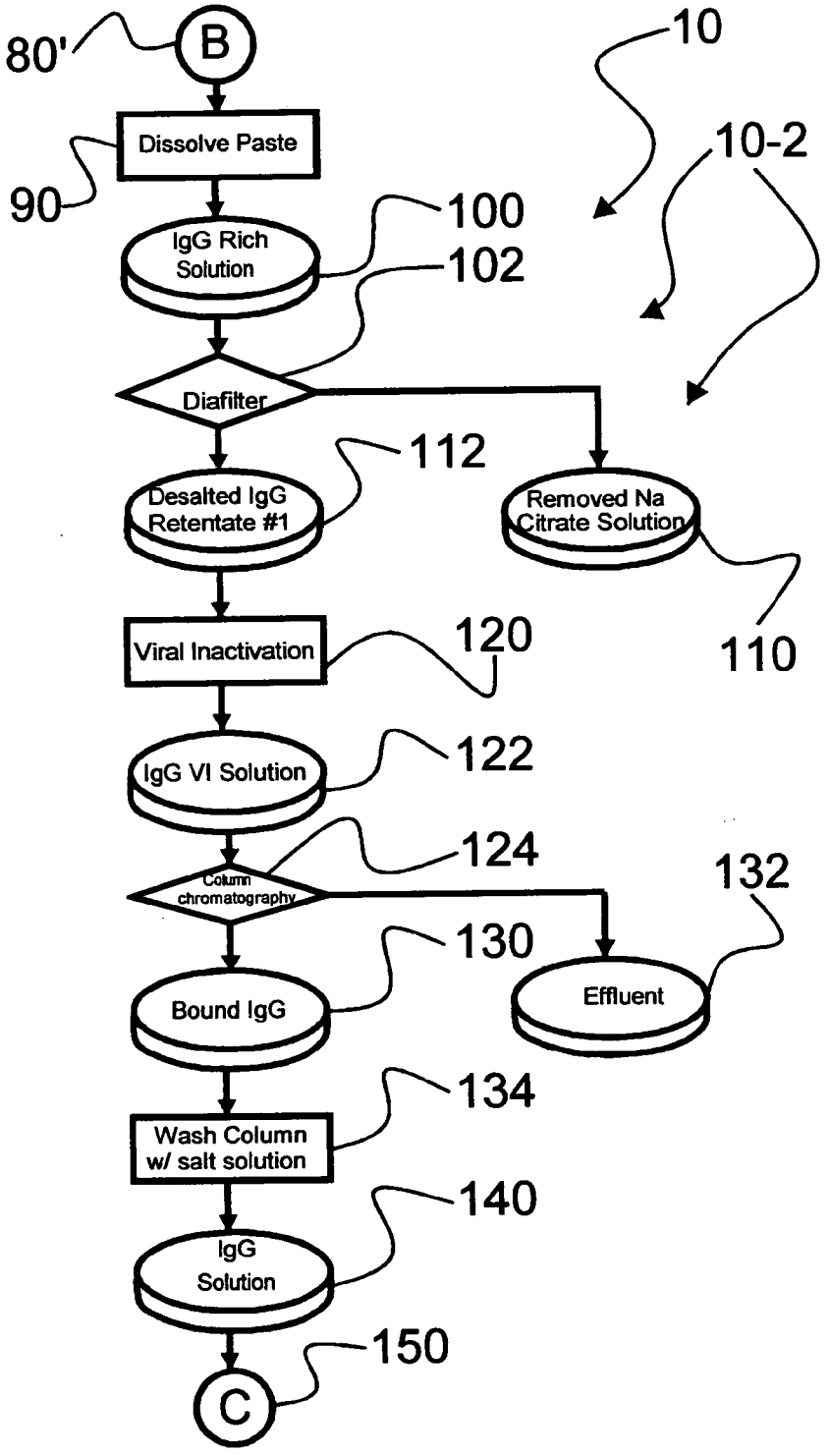
80'
B
10
10-2
Dissolve Paste
90
100
IgG Rich Solution
102
Diafilter
112
Desalted IgG Retentate #1
Removed Na Citrate Solution
110
Viral Inactivation
120
IgG VI Solution
122
124
Column chromatography
132
130
Bound IgG
Effluent
134
Wash Column w/ salt solution
140
IgG Solution
150
C

Figure 2

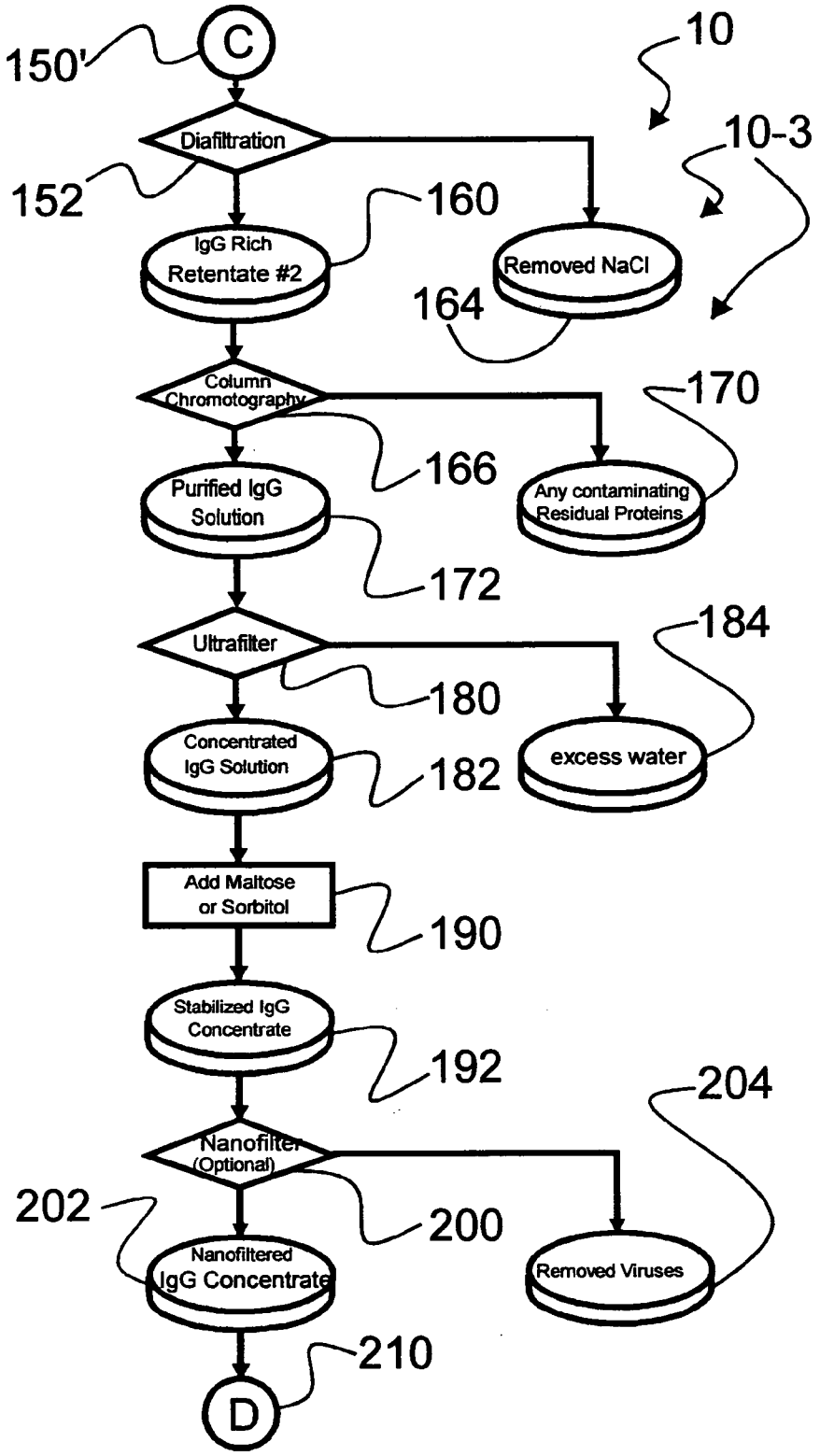
150'
C
10
Diafiltration
10-3
152
160
IgG Rich Retentate #2
Removed NaCl
164
Column Chromotography
170
166
Purified IgG Solution
Any contaminating Residual Proteins
172
Ultrafilter
184
180
Concentrated IgG Solution
182
excess water
Add Maltose or Sorbitol
190
Stabilized IgG Concentrate
192
204
Nanofilter (Optional)
202
200
Nanofiltered IgG Concentrate
Removed Viruses
210
D

Figure 3

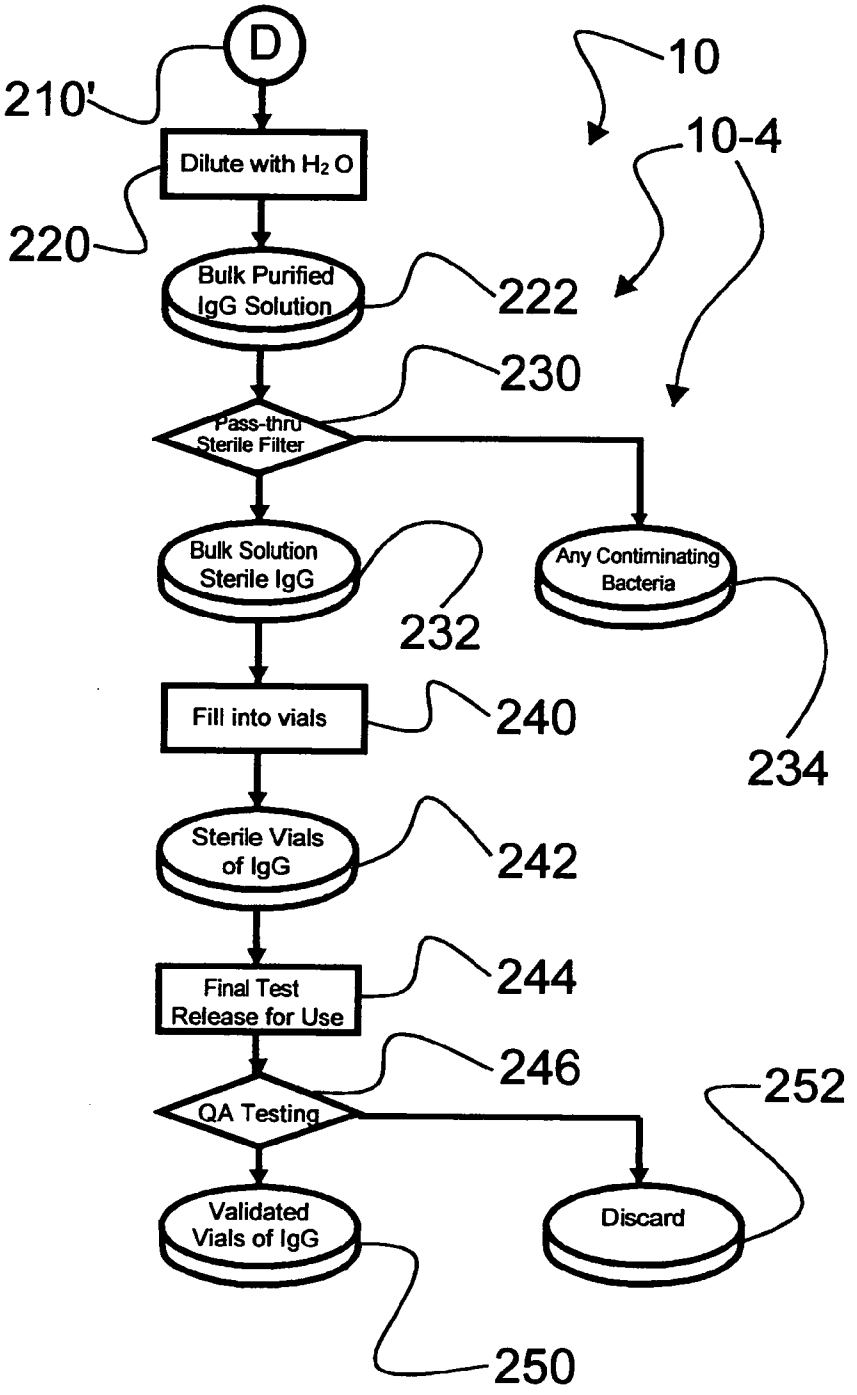
D
210'
10
10-4
Dilute with H_2O
220
Bulk Purified IgG Solution
222
230
Pass-thru Sterile Filter
Bulk Solution Sterile IgG
232
Any Contiminating Bacteria
234
Fill into vials
240
Sterile Vials of IgG
242
Final Test Release for Use
244
246
QA Testing
252
Validated Vials of IgG
Discard
250

Figure 4

ULTRA-HIGH YIELD INTRAVENOUS IMMUNE GLOBULIN PREPARATION

FIELD OF INVENTION

[0001] This invention relates generally to methods for immune serum globulin purification, and, more particularly, to methods for alcohol-free separation of immune globulin from blood plasma or other blood based material.

BACKGROUND AND DESCRIPTION OF RELATED ART

[0002] Commonly, contemporary methods for separation of immune globulins (IgG) from blood plasma or other blood based material depend upon early work by Edwin J. Cohn. As found in U.S. Pat. No. 5,177,194 issued Jan. 5, 1993 to Maria E. Sarno, et al. (SARNO), "One scheme in widespread use is the well-known Cohn fractionation method, which is based on differential precipitation using cold ethanol." Cohn et al. *J. Am. Chem. Soc.* 68, 459 (1946).

[0003] A U.S. Pat. No. 2,390,074 issued Dec. 4, 1945 to Edwin J. Cohn (Cohn) disclosed use of alcohol, acetone and dioxane as precipitants in such fractionation processes. Continued dependence upon alcohol as a precipitant is further demonstrated in U.S. Pat. No. 6,893,639 B2 issued May 17, 2005 to Joshua Levy, et al. (Levy), wherein it is stated, "The conventional industrial methods of immune globulins purification from blood plasma are based on cold ethanol fractionation which co-precipitate groups of proteins based on their isoelectric points at given alcohol concentration at sub-zero temperatures."

[0004] Cohn's work was stimulated by the need of the military for a stable solution for use as a plasma volume expander during World War II to replace lyophilized plasma. Consequently, the Cohn method focused on optimizing the process for separating the albumin fraction which provides the osmolality necessary for plasma volume expansion.

[0005] Even so, the use of alcohol precipitants is not without difficulties, as illustrated by Cohn, "Some protein precipitants, such as alcohol, have a tendency to denature many proteins with which they come in contact, the danger of denaturation increasing with concentration of the alcohol and increase in temperature. For many proteins, it has been found advisable to exercise considerable care in mixing the precipitant with the plasma or other protein solution in order to avoid denaturation of the protein." For this reason, it is considered prudent to provide an alcohol free method for blood plasma and other blood based material fractionation, including IgG purification.

[0006] In the 1970's, chromatography was found to be useful in the separation and purification of plasma proteins. Chromatography separates plasma proteins by specifically targeting unique characteristics of each, including molecular size (gel filtration), charge (ion exchange chromatography), and known interactions with specific molecules (affinity chromatography).

[0007] The use of various chromatographic methods on an industrial scale has been adopted for the isolation of small-weight, high-value proteins, such as Factor VIII, from plasma, and for the final purification of gamma globulin after separation from the plasma by Cohn, or modified Cohn methodologies. However, the separation of the large-weight, lower-value fractions such as albumin and gamma globulin, on an industrial scale has not been found to be practical.

[0008] Two U.S. Patent Applications, filed by Edward Shanbrom, having Application Numbers 20030022149 (Shanbrom '149) and 20030129167 (Shanbrom '167) filed Jan. 30, 2003 and Jul. 10, 2003, respectively, teach of use of carboxylic salts (e.g., trisodium citrate) as an agent for enhancing formation of a cryoprecititate from plasma. The method(s) of Shanbrom generally involve trisodium citrate and other citrate salts as agents for enhancing production of blood clotting factors from cryoprecipitate.

[0009] Shanbrom '149 teaches in paragraph 0009 that "It is an object of the present invention to provide enhanced yields of cryoprecipitate." Shanbrom also teaches, in paragraph 0011, that carboxylic acids are effective agents for enhancing the production of blood clotting factors from the cryoprecipitate. Shanbrom '149 notes that the addition of citrate to plasma, especially at concentrations between two and ten percent, by weight, does not appreciably denature labile proteins. Moreover, it is noted in Shanbrom '149 that citrate potentiates or enhances the killing of microorganisms by heat treatment.

[0010] Shanbrom '167 notes in paragraph 0015 that "Not only does added citrate increase the amount of cryoprecipitate, it simplifies the process by decreasing the requirement for freezing" plasma in order to harvest cryoprecipitate. Shanbrom clearly teaches use of production of a cryoprecipitate for the purpose of fractionating products from the cryoprecipitate through the use of trisodium citrate in concentrations of two to ten percent.

[0011] While Shanbrom '149 and '167 deal directly with extracting labile coagulation products from a cryoprecipitate formed through use of citrate compounds, particularly trisodium citrate, and with killing microorganisms in the cryoprecipitate using the citrate compounds, the instant invention deals directly with extracting non-labile products (e.g., albumin, gamma globulin and alpha-1-antitrypsin) from a supernatant formed through use of citrate compounds. Shanbrom neither teaches nor addresses using a supernatant in any way.

BRIEF SUMMARY AND OBJECTS OF THE INVENTION

[0012] In brief summary, this instant invention provides novel and effective methods of isolating gamma globulin from plasma and formulating it into an intravenous injectable preparation. Accordingly, this invention, which may be defined to be "an ultra-high yield intravenous immune globulin preparation," achieves higher yields of a superior quality gamma globulin by directly and expeditiously separating the gamma globulin from the plasma by means of a non-denaturing precipitant, sodium citrate.

[0013] The inventive process is for fractionating blood-based products to produce a useful, non-denatured immunoglobulin (IgG) product which involves the following critical steps:

[0014] (a) adding a first volume of sodium citrate to a quantity of blood-based material to be fractionated to form a supernatant product, free of euglobulins, the supernatant being separable from a residual paste and separating the two;

[0015] (b) performing a second fractionation step by adding a second volume of sodium citrate to the separated supernatant product, thereby forming a second separable product which is a paste and a second residual product which is supernatant, such that the products may thereafter be separated;

[0016] (c) forming a liquid dilution of the second separated paste product; and

[0017] (d) diafiltering the second separated and liquified paste product to form a low volume resulting product which is substantially free of sodium citrate and ready for processing by currently known and practiced procedures to complete production of the useful, non-denatured IgG.

[0018] In step (a), the first volume utilizes a fifty percent sodium citrate solution. The supernatant and residual paste should have a range of eleven to thirteen percent, and preferably should approximate a twelve percent concentration. If desired, the residual paste may be further fractionated into blood factors including VIII, IX, von Willebrand and fibrinogen. Separation of the products may be accomplished by centrifuging or existing methods which are well known in chemistry art.

[0019] In step (b), the second volume utilizes addition of another fifty percent sodium citrate solution. The second product is a paste and the second residual is a supernatant, both having a range of concentration of approximately twenty-one to twenty-three percent sodium citrate, which should approximate twenty-two percent. As with step (a), if desired, the residual (supernatant) may be further processed into a group of components comprising albumin and alpha-1-antitrypsin. The products may be separated by centrifuging, filtering or other methods which are well-known in the chemistry art.

[0020] In step (c), it is preferred to dilute the paste product with water having approximately four times the weight of the paste product, although other volumes of water may be judiciously selected within the scope of the invention.

[0021] In step (d) a diafiltration system with a 30 KD filtering membrane may be used to separate the sodium citrate and excess water from the resulting product to permit further processing on an industrial scale. Note, that such filtering is made facile and possible by extracting euglobulins from the supernatant in step (a). As used herein, euglobulins are defined to be those globulins which are insoluble in water, but are soluble in saline solutions. Most importantly, if euglobulins are not removed from a solution and if the ionic strength of that solution is lowered towards deionized water (e.g., in the case of the instant invention), euglobulins foul a diafiltration system, thereby rendering it unuseable.

[0022] It is well-known that sodium citrate has long been used in low concentrations during the collection, preservation and storage of blood plasma. Subsequent diafiltration after use of high concentrations of sodium citrate as a precipitant substantially reduces the ionic strength and volume of the gamma globulin solution, permitting the achievement of chromatographic purification on an industrial scale.

[0023] Following separation of gamma globulin from plasma by this method, albumin and alpha-1-antitrypsin are subsequently removed from the remaining proteins by methods available from Cohn or others. The process, according to the instant invention, enables the separation of gamma globulin without exposing it to the denaturing effects of ethanol used in the Cohn process, hence leaving the gamma globulin in a native state. The denaturing effects of alcohol include the formation of polymers, aggregates and fragments of the gamma globulin molecule. However, the use of sodium citrate stabilizes the plasma while bringing about precipitation of substantially all of the coagulation proteins, thus preventing the generation of enzyme activators and proteolytic enzymes.

[0024] The absence of the denaturing effects of ethanol, the stabilization of the plasma with sodium citrate, and the subsequent removal of coagulation proteins by means of sodium citrate results in a gamma globulin preparation which has very low anti-complementary activity.

[0025] In summary, the process of the instant invention employs high concentrations of sodium citrate combined with its subsequent removal from the gamma globulin concentrate by means of diafiltration, a technique which became practical on an industrial scale in the 1980's. Final purification of the resulting gamma globulin is then practically and effectively achieved through the use of well-established chromatographic purification techniques. The invention reduces production costs as a result of higher yields, fewer fractionation steps, shorter overall processing time, lower energy costs, and lower chemical costs. Capital costs are less because of reduced space requirements, reduced work-in-process, reduced processing time, and elimination of the explosion proof environments required for ethanol processing.

[0026] Accordingly, it is a primary object to provide an effective intravenous gamma globulin preparation at a cost which is reduced from methods in current practice.

[0027] It is an important object to provide such a method and preparation which is high-yielding.

[0028] It is a further object to provide a gamma globulin preparation which can be rapidly infused with greater patient tolerance than gamma globulin produced by methods employing alcohol.

[0029] It is therefore a principle object to provide an alcohol free method for preparing gamma globulin.

[0030] It is an object to produce gamma globulin having reduced in-process formation of polymers, aggregates, fragments, enzyme activators and proteolytic enzymes compared with similar preparations produced using traditional alcohol based methods.

[0031] These and other objects and features of the present invention will be apparent from the detailed description taken with reference to accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

[0032] FIG. **1** is a flow diagram of a critical set of initial steps associated with the process according to the instant invention.

[0033] FIG. **2** is a flow diagram disclosing a series of steps which immediately follow the steps seen in FIG. **1**.

[0034] FIG. **3** is a flow diagram disclosing those procedural steps which immediately follow the steps seen in FIG. **2**.

[0035] FIG. **4** is a flow diagram disclosing steps which immediately follow the steps seen in FIG. **3** to provide a useful product.

DETAILED DESCRIPTION OF THE ILLUSTRATED EMBODIMENTS

[0036] Reference is now made to flow path elements illustrated in FIGS. **1-4**. Generally, each rectangular box is used to illustrate a procedural step; each diamond is used to demonstrate a separation step; each elliptical cylinder designates a product resulting from a preceding procedural or separation step; and each circle is used to identify either a starting point or an off-sheet continuation path point.

[0037] Reference is now made to FIG. **1** wherein an initial portion **10-1** of an preferred IgG process flow path, generally numbered **10**, is seen. As indicated after initial starting point **20**, a volume of plasma **30** to be processed is selected for processing. It should be noted that while plasma **30** is used by example in this description of an illustrated embodiment, other blood-based products may be processed within the scope of the instant invention.

[0038] As part of procedure **40**, selected frozen plasma **30** is warmed to approximately five degrees Centigrade to form prepared plasma **50**. While five degrees is the target plasma **30** process temperature, which should be maintained throughout the following steps in process **10**, a temperature range between limits of two to eight degrees may be held within the scope of the instant invention. Plasma **30** may be used directly if not selected in a frozen state (e.g., thawed during the process of removing a cryogenic precipitate by customary methods).

[0039] A batch of sodium citrate solution is made per procedure **52** wherein a fifty percent sodium citrate solution is prepared by stirring five hundred grams of sodium citrate into six hundred milliliters of purified water. Stirring time should be thirty to sixty minutes or, alternately, until the sodium citrate is dissolved. At this point, dilute the mixture with pure water to one thousand milliliters. Add a 50% citric acid solution to the mixture until a pH of 7.0 is reached.

[0040] Preparatory to performing the first fractionation step (procedure **54**), a volume of fractionation solution to be added to plasma **30** is calculated. It is a goal that the concentration of the sodium citrate fractionation solution should be twelve percent. Also the pH of the fractionation solution should be approximately 7.0. If necessary, adjust the pH to 7.0.

[0041] The formula, (Formula I) for calculating respective volumes of fractionation solution (sodium citrate) and plasma **30** are as follows:

$$x=(C*V)/(0.5-C)$$

where:

[0042] x is desired volume of 50% sodium citrate solution;

[0043] C is a desired fractional concentration of sodium citrate; (e.g., 0.12 or twelve percent): and

[0044] V is volume of solution to be diluted, (e.g., volume of plasma **30**).

An example of a calculation by Formula I is:

[0045] For a volume (V_p) of plasma **30** of 500 liters, and the desired fractional concentration of sodium citrate is twelve percent:

$$x=(0.12*500)/(0.5-0.12)=158 \text{ liters}$$

Solving Formula I for C yields Formula II into which values of volumes of plasma **30** and sodium citrate may be inserted as follows:

$$C=(0.500*158)/(500+158)=0.12$$

[0046] For procedure **54**, over a period of approximately five minutes, add the prepared sodium citrate fractionation solution (which may be at room temperature [i.e. approximately twenty degrees Centigrade]) to plasma **30** (which has a starting temperature of five degrees Centigrade). Gently stir while adding the sodium citrate solution. Once the sodium citrate solution is completely added to plasma **30**, continue gently stirring the resulting slurry for approximately sixty minutes while reducing the slurry temperature to within a range of two to eight degrees centigrade. (The slurry should maintain pH at approximately 7.0 to 7.1.)

[0047] Upon completion of procedure **54**, centrifuge as procedure **56**. It is recommended that a flow-through centrifuge (e.g., a Westphalia Centrifuge) be used to separate component parts of the slurry into a supernatant liquid **60** and a paste **62** by normal procedures for those skilled in the art, while maintaining temperature of the slurry in the range of two to eight degrees Centigrade.

[0048] While supernatant liquid **60**, which contains virtually all of the IgG of the original plasma, is retained for further processing as an integral part of the instant inventive method, paste **62** may be further processed to recover blood factors, including Factors VIII, IX, von Willebrand and fibrinogen.

[0049] For the second fractionation phase, perform process step **64** which adds additional sodium citrate fractionation solution to supernatant liquid **60**. Enough fifty percent sodium citrate is added to liquid **60** to increase concentration of sodium citrate from twelve percent to twenty-two percent.

[0050] To calculate the volume of fifty percent sodium to be added, Formula III is provided as follows:

$$C_e=((V_{60}*C_{60})+(V_x*C_{0.50}))/(V_{60}+V_x)$$

Where C_e is the desired end concentration of sodium citrate; V_{60} is the volume of supernatant liquid **60**; C_{60} is sodium citrate concentration in supernatant liquid **60**; V_x is volume of fifty percent sodium citrate to be added; and $C_{0.50}$ is concentration of fifty percent sodium citrate (i.e. 0.50).

[0051] Note that the desired end concentration of sodium citrate in solution is 0.22 or twenty-two percent.

[0052] Solving for V_x yields Formula IV which may be used to calculate volume of sodium citrate to be added.

$$V_x=V_{60}*(C_e-C_{60})/(C_{0.50}-C_e)$$

[0053] As an example, for a volume of V_{60} of 552 liters; a concentration of C_e of 0.22; a concentration of 0.12 for C_{60}; and a concentration of 0.50 for $C_{0.50}$:

$$V_x=197 \text{ liters}$$

After adding volume V_x of sodium citrate, stir for two to four hours while retaining the temperature of this mixture between two and eight degrees Centigrade. Note that this solution will change color to a pale yellow as the additional sodium citrate is added and the mixture is stirred.

[0054] After stirring, per step **66**, centrifuge the mixture, use a continuous flow centrifuge while maintaining the temperature in the range of two to eight degrees Centigrade to separate paste **70** from supernatant **72**. The resultant supernatant (supernatant **72**) contains essentially no IgG. Virtually all of the IgG of plasma **30** is now found in paste **70**.

[0055] Reference is now made to FIG. **2** as point **80** continues to point **80'** for flow path portion **10-2** of flow path **10**. Contents of paste **70** includes IgG, other serum proteins and sodium citrate. The sodium citrate must be removed from paste **70** to permit IgG to be isolated by ion exchange chromatography. First, paste **70** is liquified using purified water (of about four times the volume of paste **70**) as step **90**. Product of step **90** is an IgG rich solution **100**. Initial conductivity of solution **100** is approximately 20 milliSiemens/centimeter (mS/cm).

[0056] Removal of sodium citrate is accomplished by continuous diafiltration using purified water as a solvent in step **102** which separates solution **100** into removed sodium citrate **110** and desalted IgG retentate **112**. Completion of step **102** is indicated when the conductivity of retentate **112** is reduced to 500-900 microSiemens/centimeter (uS/cm). For diafilitration in step **102**, a Millipore (or comparable) diafiltration system equipped with 30 KD cut-off membranes may be employed.

[0057] Viral inactivation of IgG rich retentate **112**, associated with step **120**, may be accomplished as a double viral inactivation step involving a first solvent/detergent (S/D) method, followed by an augmented S/D method. The first method employs raising the temperature of retentate **112** to approximately 27° Centigrade (temperature may range from 24-30° Centigrade). A sufficient volume of Triton X-100 or Tween 80 is then added to make a one percent solution and sufficient Tri-N-Butyl Phosphate to make a three tenths of one percent solution to make a first S/D added mixture. The first method continues by incubating the first S/D added mixture at 27° Centigrade for three hours during which time lipid enveloped viruses are inactivated. From this point, procedures currently available, inactivation and fractionation processes may be employed. However, a currently preferred process is hereafter provided for completeness.

[0058] For step **120**, a S/D concentrate may be made as follows:

[0059] Add 30 milliliters of Tri-N-Butyl Phosphate to 800 milliliters of purified water. Mix well. Add 100 milliliters of either Triton X-100 or Tween 80 to the mixed solution. Again, mix well to provide a final, mixed S/D solution. Add enough purified water to bring the total volume of the final mixed solution to 1000 milliliters. One more time, mix well. So made, the final solution is a 10× concentrate. Add 100 milliliters of this concentrate to each 900 milliliters of retentate **112** to form the first S/D added mixture.

[0060] After three hours of incubation, add, to the solution resulting from the first S/D method, sufficient formaldehyde to make a three tenths of one percent solution and sufficient phenol to make a three tenths of one percent solution to form an augmented mixture to begin the augmented method phase of step **120**. Incubate at approximately twenty seven degrees Centigrade for an additional three hours, after which time non-enveloped and enveloped viruses are inactivated.

[0061] For step **120**, an "augmented" concentrate may be made as follows:

[0062] Add 13.4 milliliters of thirty-seven and one-half percent formaldehyde solution to 900 milliliters of purified water. Mix well. Add fifty grams of phenol (reagent grade) to this mixture. Again, mix well. Add enough purified water to bring the total volume of the "augmented" preparation to one thousand milliliters. Once more, mix well. This preparations contains 50,000 parts per million each of formaldehyde and phenol (five percent of each). Measure the volume of the first S/D added mixture. Add 167 milliliters of augmented concentrate to each 833 milliliters of first S/D added mixture to form the augmented mixture.

[0063] Step **120** is completed by cooling the processed augmented mixture to a temperature of two to eight degrees Centigrade. So cooled, the augmented mixture becomes IgG virus inactivated (VI) solution **122**.

[0064] Alternatively, viruses may be removed by other methods (e.g., chromatography, nanofiltration, pasteurization), if desired.

[0065] Step **124** involves use of column chromatography to remove viral inactivation chemicals. Such may be accomplished by the following sub-steps:

[0066] 1. Set up a short, wide column with Toyopearl CM-650C resin. The Toyopearl resin is a weak cationic exchange resin used to capture IgG in solution **122** while permitting other proteins from solution **122** to flow through the column. It is important that the conductivity of solution **122** be in a range of 100 to 900 microSeimens/centimeter (uS/cm). (It is preferable that such conductivity is in a range of 400 to 600 microSeimens/centimeter.) IgG from plasma **30** binds to the exchange resin in the low ionic strength solution.

[0067] 2. Introduce solution **122** into the exchange resin at a slow rate. Collect effluent liquid from the column and measure the effluent liquid at 280 nano-meters in a one centimeter silica cuvette in a high quality spectrophotometer. (As an example, a Beckman DU-7 with a deuterium light source may be used.) It should be noted that optical density of the effluent will increase as proteins are introduced into the resin column. Phenol in the viral inactivation solution (if used) also can increase measured optical density. After all of solution **122** has passed through the resin column and sterilants are washed from the column, begin collecting the effluent when measured optical density increases from its original value. A rise in optical density is indicative of protein in the effluent. After a period, optical density drops down to a level which is indicative of little or no protein in solution. At this point, collecting may cease. At this point, it is preferable to thoroughly wash the resin with deionized water. Bound material is IgG, identified along path **10-2** as bound IgG **130**. Collected effluent from the column includes all of the protein

from plasma **30** except for IgG. This effluent is effluent solution **132**. It is recommended that serum protein electrophoresis be performed on effluent solution **132** to confirm that little or no IgG has been released into solution **132**.

[**0068**] Depending upon size of the resin column, prepare a volume of two percent solution of sodium chloride. Application of the sodium chloride is used to effect release of attached IgG from resin particles. As is well-known in chemistry art, a two percent solution is made by mixing 20 grams of sodium chloride into one liter of deionized water. Sufficient volume of two percent sodium chloride solution should be made to equal about ten times the volume of the resin column.

[**0069**] For step **134**, add the sodium chloride solution to the column, collecting effluent from the column. Concurrently, measure optical density of the effluent solution at 280 nanometers using a spectrophotometer with a one centimeter silica cuvette. Resultant optical density (OD) will be found to suddenly increase as IgG is uncoupled from the resin and delivered into the effluent. Collect all high OD measured solution. When the OD of the effluent drops to a lower (normal) range, cease collecting the solution. Resulting solution is IgG solution **140**. Note that a high OD is indicative of protein content in solution, and that solution **140** may contain small amounts of IgM and IgA, which requires further removal. In addition solution **140** contains sodium chloride which must be removed before any pure IgG can be isolated.

[**0070**] Reference is now made to continuation point **150** in FIG. **2** which continues to continuation point **150'** in FIG. **3** for flow path portion **10-3** of flow path **10**. Sodium chloride is preferably removed from solution **140** by continuous diafiltration employing a diafiltration system. Such may consist of a Millipore (or comparable) diafiltration system equipped with 30 KD cut-off membranes. As performed in step **152**, the diafiltration solvent is purified water. As may be noted, initial conductivity of solution **140** is approximately fifty milliSiemens/centimeter (mS/cm). At completion of diafiltration, conductivity is reduced to 500-900 microSiemens/centimeter (uS/cm).

[**0071**] The products of diafiltration are an IgG rich retentate **160** and removed sodium chloride **164**. It is recommended that serum electrophoresis be performed at this step in the process to quantitate protein content in retentate **160**.

[**0072**] Step **166** is a final step for purifying IgG rich retentate **160**. For step **166**, it is preferred to set up a short, wide resin column with Toyopearl QAE-550C resin. Such resin provides a strong anionic exchange for capturing other proteins in IgG rich retentate **160**, while permitting IgG in solution to flow through the column. It is important that conductivity of retentate **160** be in a range of 100 to 900 microSeimens/centimeter, and preferably, within a range of 400 to 600 microSeimens/centimeter. In this manner, IgG in retentate **160** will pass through the resin column in Step **166** without binding, while other proteins, including IgM and IgA, will bind to resin in the column and thus be removed from solution. In this manner, any contaminating residual proteins **170** are effectively separated from a purified IgG solution **172**.

[**0073**] As the process is continuous, it is recommended that IgG solution **172** be collected and the OD measured for a target 280 nm. Collect the high OD effluent solutions. When the measured OD drops, cease collecting. The pooled solution is relatively dilute.

[**0074**] The pooled solution is concentrated using step **180** via ultrafiltration. For such ultrafiltration, a hollow fiber filter may be used, or a Millipore ultrafiltration system (Pellicon) or equivalent, (10 K to 30 K dalton retentation) to concentrate to a twelve percent IgG solution **182**. Excess water **184** is removed in the process of step **180**. The resulting twelve percent concentrate should have only a trace amount of sodium chloride and the pH should be approximately seven. Conductivity should measure about 100 to 900 microSiemens/centimeter.

[**0075**] To stabilize the twelve percent IgG solution **182**, add (step **190**) a maltose or sorbitol solution to dilute the twelve percent solution to exactly ten percent. The final ten percent solution (IgG solution **192**) should contain approximately five percent maltose or sorbitol (whichever is used).

[**0076**] Optionally, to remove viruses **204** from IgG solution **192**, nanofiltration may be performed by passing the ten percent solution **192** through a virus retaining membrane (step **200**) to produce a nanofiltered concentrate **202**.

[**0077**] Reference is now made to continuation point **210'** in FIG. **4**, for flow path portion **10-4** of flow path **10**, which continues from continuation point **210** in FIG. **3**. As is standard procedure, (depending upon execution of prior option step **200**) either stabilized IgG concentrate **192** or nano-filtered IgG concentrate **202** is diluted with deionized water in step **220** to produce a bulk purified IgG solution **222**. Contaminating bacteria may be removed by passing solution **222** through a sterilizing filter in step **230** to produce a sterilized bulk IgG solution **232**. Removed contaminating bacteria **234** may be disposed of by methods currently known in the art.

[**0078**] Resulting sterile solution **232** may be filled into vials per standard procedures in step **240** to produce a lot **242** of vials of solution **232**. As required for quality assurance, final testing and inspection of lot **242** may be made in step **244** in cooperation with step **246** to produce a lot **250** of validated vials of solution **232**, with any discard **252** being removed therefrom.

Results of a Fractionation Procedure Performed According to the Instant Invention:

[**0079**] In order to show the efficacy and precision of separation of steps of the instant invention disclosed herein, the following results have been extracted from a laboratory report, dated Aug. 8, 2005.

[**0080**] In the procedure, fresh frozen human plasma was used. As is typical in such procedures, a pool was made from four to eight bags of thawed plasma (see step **40**, FIG. **1**). Commercial equipment available from Beckman-Coulter was used to evaluate various fractions as they became available. The Beckman, "Appraise", densitometer was used to scan the Beckman agarose gels for serum protein electrophoresis as part of a Paragon Electrophoresis System. For each fraction made from the pool, between three and five gel slits were loaded with five microliters of product. Results were averaged to obtain a better representative result for each fraction. Such results are found in tables provided hereafter.

[0081] The gels were electrophoresed for twenty-five minutes at 100 VDC at a pH of 8.6 and later stained with a Paragon blue stain. The Appraise densitometer was used to scan the stain-dried gels at a wavelength of 600 nanometers twice for each gel slit (ten gel slits per gel were used). An average graphic representation of the distribution of five different protein fractions, based upon density of attached dye as well as a numeric presentation of each fraction was derived. The numeric presentation was based upon a computer analysis of peaks and valleys of generated graphs at selected locations within the gel pattern as occurred between the anode and cathode on each gel. Presentation values were totaled and dye percentage was divided by the total dye amount to provide a percentage for evaluation. Note that the grand total, summing each individual blood fraction always equals one hundred percent.

[0082] As seen in Table I below, the five different protein fractions are identified as: Albumin, Alpha 1, Alpha 2, Beta and Gamma globulin. Before fractionation, a sample was removed from the pool and electrophoresed to determine the average fractional values of each fraction before beginning fractionation. Representative results for the average base pool material are listed below as percentages of whole plasma:

TABLE I

	Percentage content of each fraction				
	Albumin	Alpha 1	Alpha 2	Beta	Gamma
Base plasma	61.2	7.1	9.7	13.0	8.9

[0083] Plasma (i.e., plasma **50**) from the pool was treated with the addition of a volume of fifty percent sodium citrate to a volume of plasma to make a twelve percent solution of sodium citrate (step **54**). This mixture was stirred for sixty minutes at two to eight degrees centigrade and was then centrifuged (Step **56**) for sixty minutes at two to eight degrees centigrade. The resulting supernatant solution **60** was measured. The remaining paste **62** was weighed and put into solution by addition of deionized water. The two solutions (**60** and **62** (dissolved)) were electrophoresed using procedures cited supra, the results of which are summarized in Table II, below:

TABLE II

	Resulting percentage concentrations				
	Albumin	Alpha 1	Alpha 2	Beta	Gamma
12% Paste 62 (dissolved)	67.8	2.7	9.1	20.4	nd*
12% Supernatant 60	28.6	1.5	10.7	40.3	16.1

*nd = none detected

There was no gamma globulin found in Paste **62** (dissolved). However, there was gamma globulin found in Supernatant **60**.

[0084] Next, sufficient fifty percent solution sodium citrate was added to supernatant **60** (step **64**) to obtain a final mixture that contained twenty-two percent sodium citrate. This solution was also stirred for sixty minutes at two to eight degrees centigrade. After centrifuging (see step **66**) for sixty minutes at two to eight degrees centigrade, the resulting supernatant solution **72** was measured. The remaining paste **70** was weighed and put into solution (step **90**) by the addition of deionized water (four times weight of paste **70** in milliliters) to form IgG rich solution **100**. Samples of supernatant **72** and IgG rich solution **100** were electrophoresed by the procedure cited supra, the results of which are summarized in Table III, below:

TABLE III

	Percentage concentrations of indicated solutions				
	Albumin	Alpha 1	Alpha 2	Beta	Gamma
22% Supernatant 72	82.4	13.9	3.6	nd*	nd*
22% Sol. 100 (dissolved)	16.7	1.3	10.5	32.5	39.0

*nd = none detected

There was no gamma globulin found in supernatant **72**. However, there was gamma globulin found in IgG rich solution **100**.

The 22% supernatant fluid (which contained mostly albumin) contained essentially no beta and gamma globulin (i.e., none of such that was detected). The twenty-two percent paste solution contained the gamma globulin of interest for further fractionating to produce intravenous gamma globulin for injection. Note also that, in step **90**, time should be allowed for the paste to solvate prior to performing electrophoresis. In the experimental process, a plasma fraction between twelve percent and twenty-two percent sodium citrate was selectively isolated out for use in this isolation procedure.

[0085] To remove sodium citrate (step **102**) trapped in the twenty-two percent paste solution, a Pellicon unit was selected to diafilter solution **100**. On the average, about seven times the volume of solution **100** was required to diafilter the sodium citrate and bring conductivity of the resulting solution down to a range between 400 and 800 microSiemens/centimeter (uS/cm), before performing any column work.

[0086] After diafiltration, the desalted protein solution **112** was treated electrophoretically to determine any changes or losses as a result of diafiltration step **102**. Because sodium citrate was removed, protein movement in the electrophoretic pattern was changed somewhat through lack of interference with a contained salt. The resulting pattern was somewhat longer than a high salt concentration pattern. This elongated pattern allowed gamma globulin to separate more readily from beta globulin with a resulting increase in measured percentage as seen in Table IV, provided below:

TABLE IV

	Percentage content of diafiltrate fractions				
	Albumin	Alpha 1	Alpha 2	Beta	Gamma
Solution 112	16.6	1.5	9.9	26.9	45.2

As seen in Table IV, approximately forty-five percent of solution **112** was gamma globulin and solution **112** exhibited better separation in the electrophoresis pattern. Note, that the beta fraction went down with better separation in the electrophoresis pattern.

[0087] At this point, various currently employed methods could have been used to purify the gamma globulin in solution **112**. For that reason, the completion of this experiment could have varied from steps seen in FIGS. **2-4**. In the case of this experiment, the solution was first treated with a Solvent/Detergent solution of three hours at twenty-seven degrees Centigrade. Then an augmented sterilization solution, performed according to U.S. Pat. No. 6,881,573, titled AUGMENTED SOLVENT/DETERGENT METHOD FOR INACTIVATING ENVELOPED AND NON-ENVELOPED VIRUSES, issued to Allan L. Louderback, filed Sep. 12, 2003, was added to the mixture and further incubated for an additional three hours at twenty-seven degrees Centigrade. This dual inactivation treatment of the dialyzed diafitered solution inactivates both enveloped and non-enveloped viruses.

[0088] The sterile treated solution was transferred to an ion exchange column loaded with Toyopearl CM-650C resin. The resin adsorbed gamma globulin and allowed all of the other proteins present in solution to flow out in effluent from the column. After adding the solution to the column and adjusting the column flow to slowly drip out through the effluent end, effluent solution was measured at 280 nanometers to determine when all free proteins and sterilants had been transported through the column. Afterward, the column was washed with a two times volume of deionized purified water to assure that the effluent has a very low measured optical density at 280 nanometers.

[0089] A two percent solution of sodium chloride was then dispensed onto the top of the column and allowed to percolate through the column. Gamma globulin which was adsorbed by resin particles was freed to flow out of the column into a receiving vessel.

[0090] Collected effluent from the column with purified water (labeled as Purified Water) and effluent from the column with the two percent solution (labeled as two percent NaCl) were tested electrophoretically to show the result of selected isolation and release of gamma globulin from the resin particles. Results of this step is summarized in Table V, seen below:

TABLE V

	Resulting percentage fractions				
	Albumin	Alpha 1	Alpha 2	Beta	Gamma
Purified Water	26.0	2.7	15.2	57.0	nd*
Two Percent NaCl	nd*	nd*	nd*	1.9	98.1

*nd = none detected

Note that more than 98% of the gamma globulin was isolated in the first resin treatment. The value for beta globulin of 1.9% may be the result of an application spot when applying solution to gel. The two percent sodium chloride solution contained the gamma globulin (IgG) and, perhaps, with larger pools of plasma, may contain some IgA and IgM globulins which should be removed.

[0091] The two percent sodium chloride solution was therefore diafiltered to remove the sodium chloride for a next column treatment. Diafilitration was again performed by passing the solution though a Pellicon unit whereby the salt was removed, yielding a final product which had a conductivity of 400 to 800 microSeimens/centimeter (uS/cm). Note that it likely takes about six volumes of dionized purified water to diafilter the two percent solution.

[0092] As a final step, a column was filled with Toyopearl 560-C resin and the desalted solution was added to the top of the column and allowed to slowly percolate through the column. In this column, gamma globulin flowed right through the resin and all other proteins attached to the resin (e.g., IgA and IgM) to yield a final effluent from the column (solution **172**) that was 100% gamma globulin in an aqueous base. The effluent tested is seen in Table VI below:

TABLE VI

	Percentage content of final solution				
	Albumin	Alpha 1	Alpha 2	Beta	Gamma
Solution 172	nd*	nd*	nd*	nd*	100

*nd = none detected

[0093] The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The present embodiment is, therefore, to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are, therefore, intended to be embraced therein.

What is claimed and desired to be secured by Letters Patent is:

1. A process for blood-based material fractionation for producing a useful, non-denatured intravenous immunoglobulin (IgG) product comprising the steps of:

(a) acquiring a predetermined quantity of blood-based material for processing;

(b) preparing said quantity of blood-based material for processing;

(c) preparing for a first fractionation step by adding a first predetermined quantity of a first predetermined concentration of sodium citrate to said quantity to form a first separable product and a first residual product, said first separable product being substantially free of euglobulins;

(d) separating said first separable product from said first residual product;

(e) performing a second fractionation step by adding a second predetermined quantity of a second predetermined concentration of sodium citrate to said separated first separable product to form a second separable product and a second residual product;

(i) separating said second separable product from said second residual product;

(g) preparing said second separable product for diafiltration; and

(h) diafiltering said second separable product to form a lower volume third product substantially free of sodium citrate.

2. The process for blood-based material fractionation according to claim 1 wherein step (a) comprises selecting a quantity of plasma as the blood-based material.

3. The process for blood-based material fractionation according to claim 1 wherein step (a) comprises selecting a quantity of a blood fractionation product as the blood-based material.

4. The process for blood-based material fractionation according to claim 1 wherein step (b) comprises thawing said quantity of blood-based material.

5. The process for blood-based material fractionation according to claim 1 wherein step (c) comprises adding a fifty percent sodium citrate solution to said quantity of blood-based material.

6. The process for blood-based material fractionation according to claim 5 wherein step (c) comprises forming said first separable product as a first supernatant solution, having a first predetermined sodium citrate concentration, in combination with said first residual product as a first paste residual product, having the same predetermined sodium citrate concentration.

7. The process for blood-based material fractionation according to claim 6 wherein step (c) comprises forming said first separable product as a first supernatant solution, having a twelve percent sodium citrate concentration, in combination with said first residual product as a first paste residual product, having the same twelve percent sodium citrate concentration.

8. The process for blood-based material fractionation according to claim 6 wherein step (c) comprises forming said first separable product as a first supernatant solution, having a sodium citrate concentration in a first range between ten and fourteen percent, in combination with said first residual product as a first paste residual product, having a sodium citrate concentration in the same first range.

9. The process for blood-based material fractionation according to claim 6 wherein step (c) comprises forming said first separable product as a first supernatant solution, having a sodium citrate concentration in a second range between eleven and thirteen percent, in combination with said first residual product as a first paste residual product, having a sodium citrate concentration in the same second range.

10. The process for blood-based material fractionation according to claim 1 comprising an additional step of fractionating said first residual product into a group of products comprising blood factors comprising Factors VIII, IX, and vonWillebrand, and fibrinogen.

11. The process for blood-based material fractionation according to claim 1 wherein step (d) comprises separating said first separable product from said first residual product by centrifuging.

12. The process for blood-based material fractionation according to claim 11 wherein step (d) comprises removing said first separable product as a first supernatant fluid.

13. The process for blood-based material fractionation according to claim 12 wherein step (e) comprises adding a fifty percent sodium citrate solution to said first supernatant fluid.

14. The process for blood-based material fractionation according to claim 13 wherein step (e) comprises forming a second paste product, having a second predetermined sodium citrate concentration, and a second supernatant product having the same second predetermined sodium citrate concentration.

15. The process for blood-based material fractionation according to claim 14 wherein step (e) comprises forming a second paste product, having a twenty-two percent sodium citrate concentration, and a second supernatant product having the same twenty-two percent sodium citrate concentration.

16. The process for blood-based material fractionation according to claim 14 wherein step (e) comprises forming a second paste product, having a sodium citrate concentration within a range of twenty to twenty-four percent, and a second supernatant product having the same range of twenty to twenty-four percent sodium citrate concentration.

17. The process for blood-based material fractionation according to claim 14 wherein step (e) comprises forming a second paste product, having a sodium citrate concentration within a range of twenty-one to twenty-three percent, and a second supernatant product having the same range of twenty-one to twenty-three percent sodium citrate concentration.

18. The process for blood-based material fractionation according to claim 14 comprising an additional step of fractionating said second residual product into a group of products comprising albumin and alpha-1-antitrypsin.

19. The process for blood-based material fractionation according to claim 14 wherein step (f) comprises separating said second separable product from said second residual product by centrifuging.

20. The process for blood-based material fractionation according to claim 14 wherein step (f) comprises separating said second separable product from said second residual product by filtering.

21. The process for blood-based material fractionation according to claim 14 wherein step (f) comprises separating said second separable product as a paste of a third predetermined concentration having a resulting weight.

22. The process for blood-based material fractionation according to claim 21 wherein step (g) comprises forming a liquid dilution of said separable paste product of a third predetermined concentration by combining said resulting volume of said separable paste product with a volume of water having four times the resulting weight.

23. The process for blood-based material fractionation according to claim 22 wherein step (h) comprises passing the liquid dilution over a filtering membrane for the purpose of separating a portion of the third product from the added sodium citrate to provide the substantially free of sodium citrate third product.

24. The process for blood-based material fractionation according to claim 22 wherein step (h) comprises passing the liquid dilution over a 30 KD diafiltering membrane for the purpose of separating a portion of third product from the added sodium citrate to provide the substantially free of sodium citrate third product.

25. The process for blood-based material fractionation according to claim 23 wherein step (h) comprises testing conductivity of the third product for a first predetermined conductivity prior to diafiltration and testing the substantially free of sodium citrate third product for a second predetermined conductivity.

26. The process for blood-based material fractionation according to claim 25 wherein step (h) comprises testing the

conductivity to assure third product conductivity is in the range of 500 microSiemens/cm (uS/cm) plus or minus 400 microSiemens/cm (uS/cm).

27. The process for blood-based material fractionation according to claim 25 wherein step (h) comprises testing the conductivity to assure the substantially free of sodium citrate third product conductivity is in the range of 500 microSiemens/centimeter (uS/cm) plus or minus 400 microSiemens/centimeter (uS/cm).

28. The process for blood-based material fractionation according to claim 27 comprising the following additional step:

(i) adding a predetermined volume of a predetermined concentration of detergent selected from a group of detergents comprising a solvent detergent and an augmented solvent detergent for the purpose of inactivating viruses contained in the substantially free of sodium citrate third product to produce an IgG viral inactivated solution.

29. The process for blood-based material fractionation according to claim 1 comprising a following additional step of cooling the IgG inactivated solution to a range of two to eight degrees Centigrade.

30. The process for blood-based material fractionation according to claim 29 comprising the following additional steps:

(j) performing column chromatography on the IgG viral inactivated solution to produce a resin bound IgG and an effluent;

(k) separating the resin bound IgG from the effluent; and

(l) washing the column resin bound IgG with a predetermined concentration of sodium chloride to produce an IgG rich solution free of viral inactivation chemicals.

31. The process for blood-based material fractionation according to claim 30 wherein step (j) comprises using a weak cationic exchange resin for the purpose of purifying the IgG rich solution free of viral inactivation chemicals to produce a purified IgG solution.

32. The process for blood-based material fractionation according to claim 30 wherein step (j) comprises using a Toyopearl CM-650C resin.

33. The process for blood-based material fractionation according to claim 30 wherein step (j) comprises testing conductivity of the IgG rich solution free of viral inactivation chemicals to assure said solution has a conductivity in the range of 100-900 microSiemens/centimeter (uS/cm).

34. The process for blood-based material fractionation according to claim 30 comprising a following additional step:

(m) concentrating the purified IgG solution to a predetermined concentration of IgG solution by ultrafiltering said solution.

35. The process for blood-based material fractionation according to claim 34 wherein step (m) comprises concentrating the purified IgG solution to a twelve percent concentration of IgG solution by ultrafiltering said solution.

36. The process for blood-based material fractionation according to claim 34 comprising a following additional step:

(n) stabilizing the predetermined concentration of IgG solution by adding a stabilizing chemical selected from a group of chemicals including maltose and sorbitol to produce a stabilized IgG concentrated solution.

37. The process for blood-based material fractionation according to claim 36 comprising a following additional step:

(o) if desired, nanofiltering the stabilized IgG concentrated solution to produce a nanofiltered stabilized IgG concentrated solution.

38. The process for blood-based material fractionation according to claim 37 comprising a following additional step:

(p) using sterile water for injection, diluting the stabilized IgG concentrated solution to a predetermined concentration of bulk purified IgG.

39. The process for blood-based material fractionation according to claim 36 comprising a following additional step:

(q) using sterile water for injection, diluting the stabilized IgG concentrated solution to a concentration of ten percent bulk purified IgG.

40. The process for blood-based material fractionation according to claim 38 comprising a following additional step:

(r) filtering for the purpose of sterilizing the bulk purified IgG concentrated solution.

41. The process for blood-based material fractionation according to claim 40 comprising a following additional step:

(s) dispensing the sterilized bulk purified concentrated IgG solution into sterile vials for distribution.

42. The process for blood-based material fractionation according to claim 41 comprising a following additional step:

(t) testing samples of contents of the sterile vials for safety and efficacy and, when vials pass predetermined testing criteria, releasing the vials to distribution channels.

43. The process for blood-based material fractionation according to claim 1 wherein a further step following step (f) processes said second residual product to separate out albumin and alpha-1-antitrypsin.

44. A method for separating euglobulins from blood elements being fractionated from blood-based material for the purpose of performing a subsequent diafiltration procedure on the separated blood elements comprising the steps of:

(a) acquiring a predetermined volume of blood-based material for processing;

(b) preparing said volume of blood-based for processing;

(c) preparing for a first fractionation step by adding a first predetermined volume of a first predetermined concentration of sodium citrate to said sample to form a first separable supernatant product and a first residual product, said first separable supernatant product being substantially free of euglobulins.

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