BLOOD COMPONENTS AND REGENERATIVE MEDICINE

Original article

Process steps for the fractionation of immunoglobulin (Ig) G depleted of IgA, isoagglutinins, and devoid of in vitro thrombogenicity

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Background - Plasma-derived immunoglobulins (IgG) are essential medicines that are in worldwide shortage, especially in low- and middle-income countries. Optimised manufacturing processes can increase supply. We evaluated various new process steps for IgG fractionation.

Material and methods - A crude, worst-case, IgG intermediate obtained by caprylic acid fractionation of cryoprecipitate-poor plasma was used as starting experimental material. It was processed inline by Fractogel® (Merck) TMAE anion-exchanger to deplete IgA and IgM, Eshmuno® P (Merck) anti-A and anti-B affinity chromatography to remove anti-A and anti-B isoagglutinins, 0.3% TnBP-1% Triton X-100 (S/D) treatment, C18 chromatography for removal of S/D agents, and single-pass tangential flow filtration (SPTFF) concentration to 20%. Quality, safety, and recovery were evaluated at small and pilot scales to assess purity, removal of IgA, IgM isoagglutinins, S/D agents, thrombogenic factors, and lack of toxicity in a cell model.

Results - The starting IgG intermediate contained approximately 90% IgG, IgA, and IgM and 10% albumin. Fractogel® TMAE, equilibrated in 25 mM sodium acetate-pH 6.0 and loaded with up to 225 mg of IgG/mL, could remove IgA and IgM, with over 94% IgG recovery with preserved sub-class distribution in the flow-through. Sequential Eshmuno®-P anti-A and anti-B columns efficiently removed isoagglutinins. The C18 packing, used at up to 17 mL of S/D-IgG solution per mL, removed TnBP and Triton X-100 to less than 1 and 2 ppm, respectively. The 20% purified IgG was devoid of activated factor XI and thrombin generation activity.

Discussion - This purification sequence yields a >99% pure, 20% (v/v) IgG product, depleted of IgA, isoagglutinins, and thrombogenic markers, and should be implementable on various IgG intermediates to help improve the supply of immunoglobulins.

Keywords: *plasma, IgG, IgA, anti-A, anti-B, factor XI, solvent-detergent.*

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INTRODUCTION

Over the last twenty years, human polyvalent immunoglobulin G (IgG) has become the leading blood-derived medicine manufactured by the human plasma fractionation industry[1]. Administering polyvalent IgG by intravenous (IV) or subcutaneous (SC) routes

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on at least a monthly basis is a vital substitutive therapy for patients suffering from primary immunodeficiency who are susceptible to recurrent viral or bacterial infections and associated organ damage[2-6]. IgG is also a therapy for secondary immunodeficiencies resulting from haematological malignancies, or subsequent to stem cell transplantation[7]. In addition, the use of high-dose polyvalent IgG as immunomodulators in neurological, haematological and dermatological autoimmune and inflammatory diseases has increased sharply in the last ten years following demonstration of efficacy by clinical trials[8-10]. In addition, hyperimmune IgG purified from plasma of donors with high antibody titres against particular haematological (RhD), bacterial (tetanus toxin), or viral (rabies, hepatitis B, etc.) antigens, are also essential therapies in human medicine. Finally, passive immunisation by "convalescent" IgG fractionated from the plasma of donors who have recovered from emerging infections, is a first therapeutic line for prophylaxis or treatment of patients[11-13]. For example, convalescent COVID-19 IgG are currently under clinical evaluation in the prevention or treatment of SARs-CoV-2 infections[14]. The therapeutic importance of polyvalent or hyperimmune IgG in any national healthcare system is reflected by their listing as "essential medicines" by the World Health Organization (WHO)[15].

Although in the years to come some of the indications of IgG to treat auto-immune, inflammatory or infectious diseases may eventually be replaced by monoclonal antibodies or alternative therapies[6,16], new clinical indications of plasma-derived IgG continue to be explored[17]. The growing needs for immunoglobulins further expose the world to shortages[18], and these have a serious impact on low- and middle-income countries (LMIC) where the lack of product affects immunodeficient patients in need[19].

Developing robust plasma fractionation steps while improving IgG quality and recovery remains a main focus of plasma fractionators and industry suppliers. Over the last 30 years, the manufacturing process of IgG has continuously evolved to enhance product safety margins, while also improving productivity and recovery[20]. For example, robust pathogen inactivation or removal treatments, such as solvent/detergent (S/D)[21] and nanofiltration[22,23] have been implemented to almost completely eliminate the risks of transmission of blood-borne viruses[24,25]. Improved process control and polishing manufacturing steps have avoided contamination by proteolytic enzymes, including prekallicrein activator[20] or activated coagulation factor XI (FXIa), thereby decreasing the risks of kinin-related hypotensive effects[26,27] and thrombo-embolic events in IVIG recipients[26-29]. Chromatographic purification steps have been developed to remove IgA that can cause anaphylactic shock in IgA-deficient patients with anti-IgA antibodies[30]. Caprylic acid treatment has been introduced to partially replace ethanol fractionation steps or S/D treatment, and improve product quality, virus safety and recovery[31,32]. More recently, dedicated immunoaffinity chromatographic steps have been engineered to remove anti-A and anti-B isoagglutinins[31,33] and to lower occurrence of haemolytic adverse events in patients, also alleviating the need for plasmapheresis donation isoagglutinin screening[34,35]. Finally, concentration of liquid IgG formulations to 15-20% have been developed to permit SC administration[36] that facilitates home treatments, and improves patient satisfaction and quality of life[5,37].

Suppliers to the biotechnology industry are playing an important role in exploring new purification approaches to facilitate plasma processing and purification[38]. We report here the evaluation of several purification steps using, as worst-case scenario, a crude immunoglobulin intermediate obtained by caprylic acid precipitation of cryoprecipitate-poor plasma (CPP). The evaluated steps were: (a) removal of IgA by anion-exchange chromatography; (b) removal of anti-A and anti-B isoagglutinins by immune-affinity capture; (c) S/D treatment and removal of virus inactivation agents by hydrophobic interaction chromatography; and (d) concentration of the purified IgG to 200 mg/mL (20%). These scalable purification steps should be readily implementable on various plasma intermediates to manufacture S/D-treated concentrated polyvalent, hyperimmune, or convalescent immunoglobulins depleted of IgA, IgM, isoagglutinins, and thrombogenic factors.

MATERIALS AND METHODS

The fractionation scheme is shown in **Figure 1** and quality control steps are summarised in the experimental design in *Online Supplementary* **Figure S1**.

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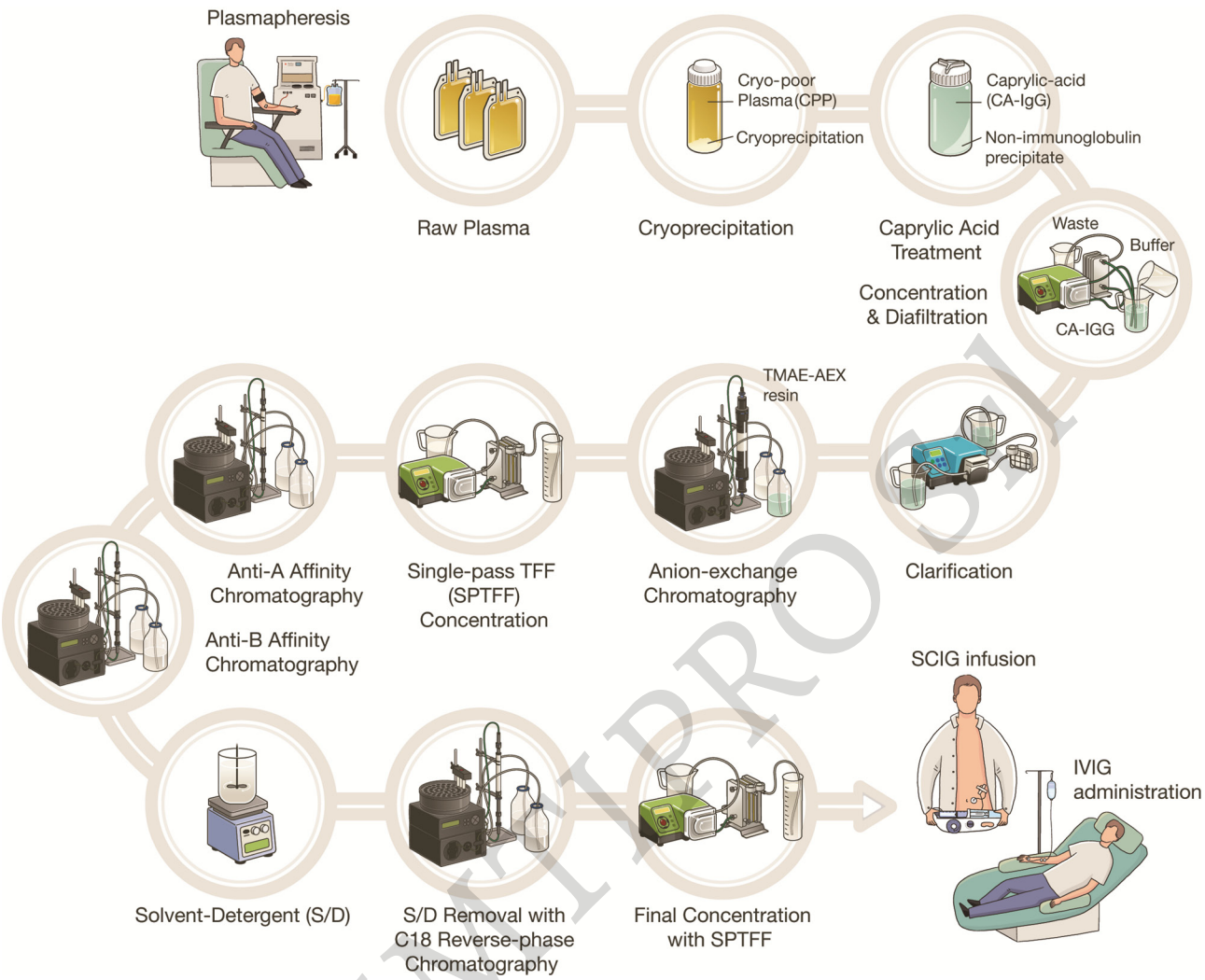


Figure 1 - IgG fractionation scheme
Graphical representation of the process steps used to isolate immunoglobulin G from human plasma using the inline fractionation process described in this study.

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Cryoprecipitation and caprylic acid fractionation

Anticoagulated human plasma was collected from regular whole blood or apheresis donors at the Taipei Blood Center, with the approval of the Institutional Review Board of Taipei Medical University (TMU-JIRB N201802052). Plasma was frozen within 8 hours (h) after collection, and delivered to our laboratory where it was maintained at −20°C or colder until use. The caprylic-acid IgG intermediate (CA-IgG) used as starting material was prepared as described in our previous research[38-40], i.e. using plasma batches initially of up to 2 L, followed by pooling to approximately 10 L for pilot-scale verification and optimisation. Plasma was first subjected to a cryoprecipitation step by thawing at 2-4°C for approximately 16 h and then centrifuged at 6,000 × *g* for 30 minutes (min) at 2-4°C (Beckman Avant J-25 Centrifuge, Beckman Coulter, Brea, CA, USA) to recover the CPP supernatant. Caprylic acid (Merck, KGaA, Darmstadt, Germany) was then slowly added to CPP under constant rapid stirring until a final concentration of 5% (v/v) and pH 5.5±0.2 was achieved, followed by mild mixing at 22±2°C for 60 min[39]. The liquid phase containing the immunoglobulins was recovered by centrifugation at 10,000 g for 30 min at 22±2°C, and filtered by Milligard PES 1.2/0.2 µm nominal Opticap capsule (Merck) followed by sterile filtration using Durapore® 0.22 µm (Merck). The clear, slightly blue-green, supernatant was diafiltered

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using a Cogent® microscale TFF system (Merck) against a 25 mM sodium acetate buffer at either pH 5.7, 6.0, or 6.3 for laboratory-scale experiments to optimise the downstream processing steps. For pilot-scale experiments, pH 6.0 was used for diafiltration and the IgG was concentrated to half its initial volume. It was then passed through a depth filter (Millistak+® HC Pod Depth Filter, A1HC µPod, Merck) followed by a Millipore Express® SHC 0.5/0.2 µm (Opticap® Capsule Filter, Merck) for clarification, and bioburden and particle removal.

IgA and IgM removal using Fractogel® TMAE

Optimisation of chromatographic conditions

The dialysed CA-IgG was chromatographed on a tri-methyl ammonium ethyl (TMAE)-Fractogel® (Merck) strong anion-exchanger packed in 1 mL MiniChrom column (Merck) equilibrated with 5 column volume (CV) of a 25 mM sodium acetate at pH 5.7, 6.0, or 6.3 and run at a linear flow-rate of 180 cm/h. Influence of protein loading and pH on IgG, IgA and IgM content in the flow-through and wash fractions were determined. After each cycle the column was washed by a 500 mM sodium acetate buffer (3 CV) to remove bound proteins, then regenerated in 1.5 M NaCl, 250 mM sodium acetate, pH 4.5 (2 CV), and finally sanitised with 0.5 M NaOH (3CV) using a 10 min residence time and 30 min contact time. Between cycles, the column was stored in 20% ethanol containing 150 mM NaCl. Conditions found optimal for IgA and IgM removal were confirmed in ten consecutive chromatographic runs. Robustness of IgG purification and recovery, preservation of IgG subclass distribution, and IgA and IgM removal were further tested over 200 consecutive cycles using a 1 mL Fractogel® TMAE (M) pre-packed column. Each cycle included CA-IgG injection, washing, cleaning, and sanitising steps, as described above, to mimic industrial manufacturing practices. The IgG flow-through obtained every ten cycles was saved and used for analysis, as described below.

Pilot-scale conditions

Pilot-scale experiments were performed using 8-10 L of plasma, yielding approximately 7-8 L of CA-IgG that were concentrated to half its volume, diafiltered and sterile-filtered (see below). For each cycle, 666 mL of CA-IgG was injected into 119 mL of Fractogel® TMAE packed in a Merck Superformance 300-26 column (height: 300 mm; cross-sectional area: 5.3 cm^2; diameter: 2.6 cm) (Merck) at a flow-rate of 23.8 mL/min using the chromatographic conditions and buffer pH pre-defined at small scale.

Anti-A and anti-B removal

The Fractogel® TMAE IgG flow-through was chromatographed first through a Vantage L column packed with a hydrophilic polyvinylether core resin functionalised with trisaccharide blood group antigen A (Eshmuno® P Anti-A affinity chromatography resin; Merck). The flow-through was directly run through a second column packed with the same core resin functionalised with trisaccharide blood group antigen B (Eshmuno® P Anti-B affinity chromatography resin, Merck). Preliminary experiments were carried out on a laboratory scale to evaluate the conditions (feed/resin loading ratio, linear flow-rate, residence time) to provide efficient removal of anti-A and anti-B isoagglutinins. Both columns were equilibrated, washed and sanitised with the same buffers as for Fractogel® TMAE column. The Eshmuno®-P resins were stored in 20% ethanol.

Solvent-detergent treatment and octadecyl reverse phase hydrophobic chromatography

The IgG flow-through of the Eshmuno® P Anti-B column was virus-inactivated by incubation at 25±1°C and under mild mixing with a mixture of tri-n-butyl-phosphate (TnBP; Merck) and Triton X-100 (Merck) at respective final concentrations of 0.3 and 1% (v/v). Laboratory-scale experiments were carried out to determine the capacity of a C18 LiChroprep® RP-18 (40-63 µm, EMD Millipore Corporation) to adsorb TnBP and Triton X-100. The S/D-treated purified IgG fraction was injected under continuous mixing onto a Vantage column packed with 1 mL-C18 (LiChroprep® RP-18, 40-63 µm, EMD Millipore Corporation) and the flow-through recovered in separate fractions to assess the TnBP and Triton X-100 content. For pilot-scale experiments, the S/D treated IgG was injected at a ratio of 973 mL per 160 mL of C18 to absorb the S/D agents and recover the IgG in the flow-through. The toxicity of the flow-through was tested by cell assays, as described below.

Single-pass tangential flow filtration

Two single-pass tangential flow filtration (SPTFF) steps were performed throughout the process. First SPTFF was

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performed before Eshmuno® P Anti-A step to reduce the loading volume and time by connecting three 0.11 m^2 30 kDa MWCO, C screen Pellicon® 3 cassettes (Cat. No. P3B030A) in serial. Optimisation excursion was performed to determine the optimal TMP at 11-12 pound per square inch (psi), with feed flux 0.57 LMM. Conversion rate was 83% to achieve six times concentration. A second SPTFF was performed at the final concentration stage of the purified IgG to reach the 20% (w/v) targeted concentration. SPTFF was set up by connecting three 88 cm^2 Pellicon® 3 cassettes in serial, 30 KDa MWCO, polyethersulfone membrane, with D screen for high concentration ultrafiltration (Merck P3B030D). Optimisation excursion determined the optimal TMP at 9 psi, with feed flux 0.1 LMM. Conversion rate was 80% to reach five times concentration.

Prefiltration and sterile filtration

Various prefiltrations and sterile filtrations had been performed throughout the process (**Figure 1**) to ensure an acceptable minimal bioburden level and process stability. The filters tested including Milligard Express® SHF 0.2 µm (PES), Millistak® + A1HC, Durapore® CVGL 0.22 µm (PVDF), Millipore Express® SHC 0.5/0.2 µm (PES) (all from Merck). Standard filter sizing methods were used including Vmax™ with constant pressure and Pmax™ with constant feed flux, to evaluate filter combination performance. The optimised filtration train was selected for further experiments based on filter throughput and filtrate quality.

Quality assays

Methods used to assess protein composition, thrombin generation, presence of FXIa, residual amount of TnBP and Triton X-100, and cell toxicity are described in the *Online Supplementary Content*.

RESULTS

Optimisation of Fractogel® TMAE

The IgG intermediate prepared from 5% caprylic acid-pH5.5 treatment of cryoprecipitate-poor plasma contained a mean of 10±1 g/L total protein, 7.46±0.11 g/L IgG, 1.34±0.04 g/L IgA, 0.19±0.02 g/L IgM, and 1.33±0.58 g/L albumin. Using a column packed with 2.5 mL of Fractogel® TMAE, we first defined, the maximum protein loading and the impact of pH for maximum IgA and IgM binding, and optimal recovery and purity of the IgG in the flow-through. A typical elution profile is shown in *Online Supplementary* **Figure S2**. Flow-through samples were taken at regular elution intervals and assessed. Data (**Figure 2**) showed that the buffer pH (pH5.7 [**Figure 2A**], 6.0 [**Figure 2B**] or 6.3 [**Figure 2C**]) had no detectable impact on IgG purity or recovery in the flow-through, IgA or IgM binding, or loading conditions. For further confirmation of process conditions, a loading of 12.5 mL CA-IgG and pH 6.0 buffer were selected to provide optimal IgA and IgM elimination as well as purity and recovery of IgG. To assess the robustness of these conditions, we then performed ten consecutive cycles. **Figure 2D** shows a comparison of the protein composition from every cycle from cycle 1 (C1) to cycle 10 (C10). The mean composition of IgG, IgA and IgM was 96.76±0.28%, 2.99±0.2%, and 0.25±0.12%. To fully assess the robustness of these conditions, we then performed 201 cycles. IgG in each flow-through of 201 cycles had a mean purity ranging from over 94.22±1.87% to 96.16±7.05%, and 96.89±7.06% recovery, confirming consistent binding of IgA and IgM on the resin (data not shown). The composition of IgG, IgA and IgM in each flow-through of 201 cycles is shown in *Online Supplementary* **Figure S3**. Subclass distribution (*Online Supplementary* **Figure S4**) was also consistent, with a proportion of 61.83±3.35%, 35.35±3.32%, 1.42±0.09%, and 1.39±0.08% for sub-classes 1, 2, 3, and 4 in the flow-through, respectively, compared to approximately, 61.43%, 34.49%, 1.46%, and 2.62% in the feed of IgG fraction.

Optimisation of Eshmuno® P anti-A and anti-B chromatography

Small and pilot-scale experiments (**Figure 3**) using IgG Fractogel® TMAE flow-through with variable anti-A and anti-B titres showed a consistent decrease of at least 8- and 16-fold in anti-A and anti-B isoagglutinin titres following Eshmuno® P anti-A and Eshmuno® P anti-B affinity chromatography.

Optimisation of C18 hydrophobic interaction chromatography

A volume of 20 mL of IgG solution was injected onto a column packed with the 1 mL of C18 material. The flow-through collected at regular intervals showed that the S/D agents were undetectable at a ratio of 17 mL S/D-treated IgG/mL of C18. Furthermore, the viability assay performed using 3T3 cells (*Online Supplementary* **Figure S5**) showed a lack of cellular toxicity of the

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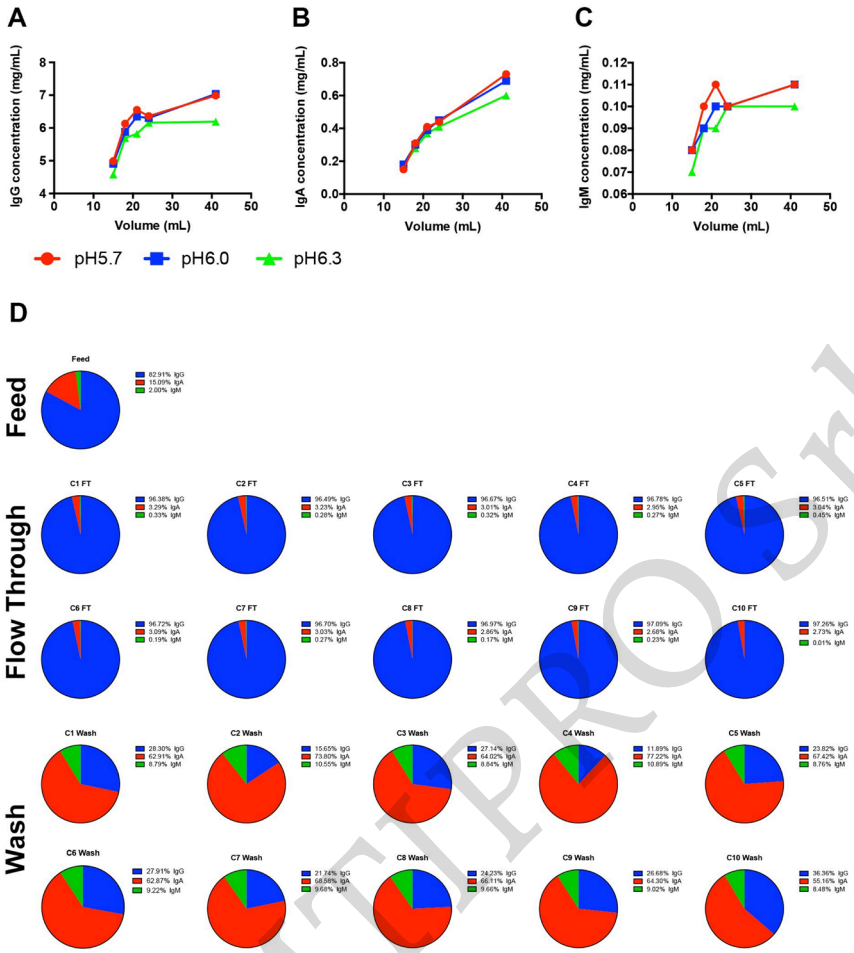


Figure 2 - Results of loading experiments and pH effect
Up to 40 mL of the caprylic acid purified Ig was continuously loaded onto a 1 mL column packed with Fractogel® TMAE equilibrated with 25 mM sodium acetate at pH 5.7, 6.0, or 6.3 and run using a linear flow-rate of 180 cm/h; see Figure 1 for conditions. Samples of the flow-through were collected at different time points. The bound proteins were eluted with 500 mM sodium acetate wash buffer. Concentrations of IgG (A), IgA (B), and IgM (C) in the flow-through were assessed. The percentage of IgG, IgA and IgM in the each collected fractions was analysed (D). Ig: immunoglobulin.

IgG flow-through up to a ratio of 17 mL per mL of C18 packing material, corresponding to a residual amount of S/D agents close to 1 ppm, as deducted from the data obtained with the standard curve. Indeed, NIH-3T3 cells treated with 2 ppm S/D-spiked IgG (standard curve) started to evidence a 4.3% decrease in viability. Increasingly lower cell viability was found when the injection volumes were 18 mL and 19 mL, indicating the presence of toxic doses S/D agents in the flow-through. Complete cell death due to presence of increasingly toxic amounts of the S/D agents was found at ratios equal to or above 20 mL of S/D-treated IgG/mL of C18 material.

Single-pass tangential flow filtration

The intermediate IgG sample was concentrated from 10 mg/mL to 58 mg/mL by first SPTFF step, and the final IgG sample was concentrated from 42 mg/mL to 198 mg/mL by second SPTFF step, successfully reaching the target close to 20% (w/v) IgG final concentration. A simulation run over 300 min maintained a steady conversion rate to reach 200 mg/mL target concentration (**Figure 4**). **Figure 5** shows the SDS-PAGE profile under non-reducing (**Figure 5A**) and reducing (**Figure 5B**) conditions, and the zone electrophoresis data over the purification steps, both illustrating the role of Fractogel® TMAE step in the enhancement of IgG purity.

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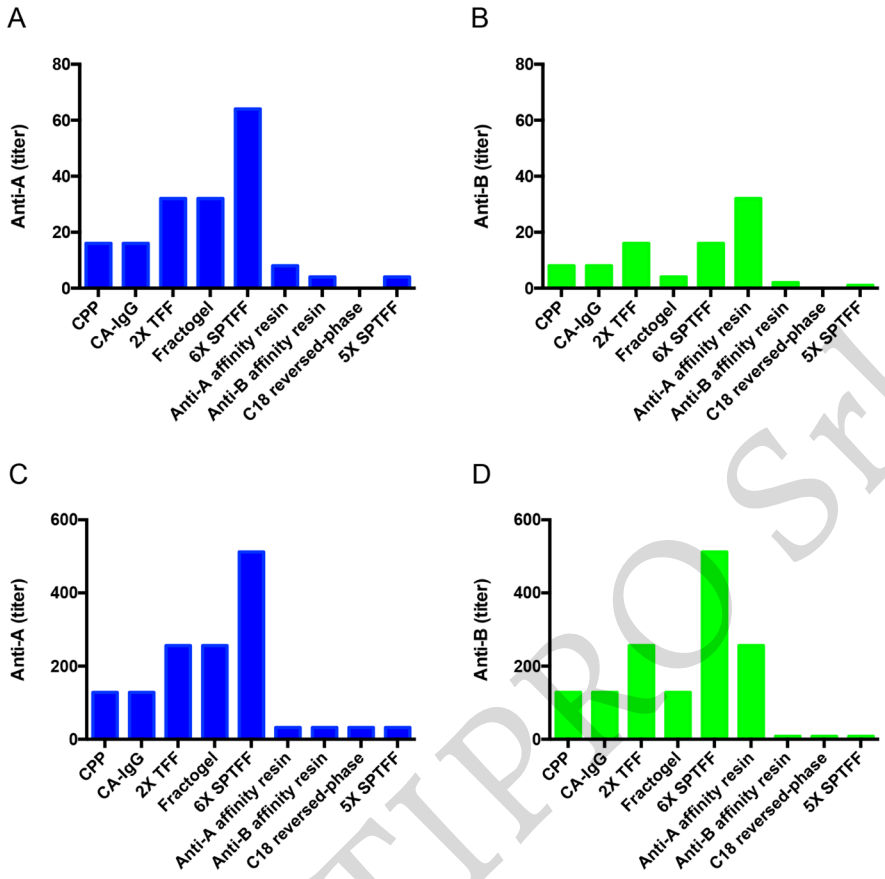


Figure 3 - Optimisation of the Eshmuno®-P anti-A and Eshmuno®-P anti-B affinity chromatographic steps to deplete anti-A and anti-B isoagglutinins

These columns were equilibrated in 25 mM sodium acetate at pH 6.0. The IgG flow-through was collected and analysed to assess titres of anti-A and anti-B isoagglutinins and its reduction over Eshmuno®-P Anti-A, and Eshmuno®-P Anti-B resins. The Anti-A titre (A and C) and Anti-B titre (B and D) of each fraction during all purification steps in two consecutive pilot-scale batches. CA-IgG: caprylic acid-purified immunoglobulin G; CPP: cryoprecipitate-poor plasma; SPTFF: single-pass tangential flow filtration; TFF: tangential flow filtration.

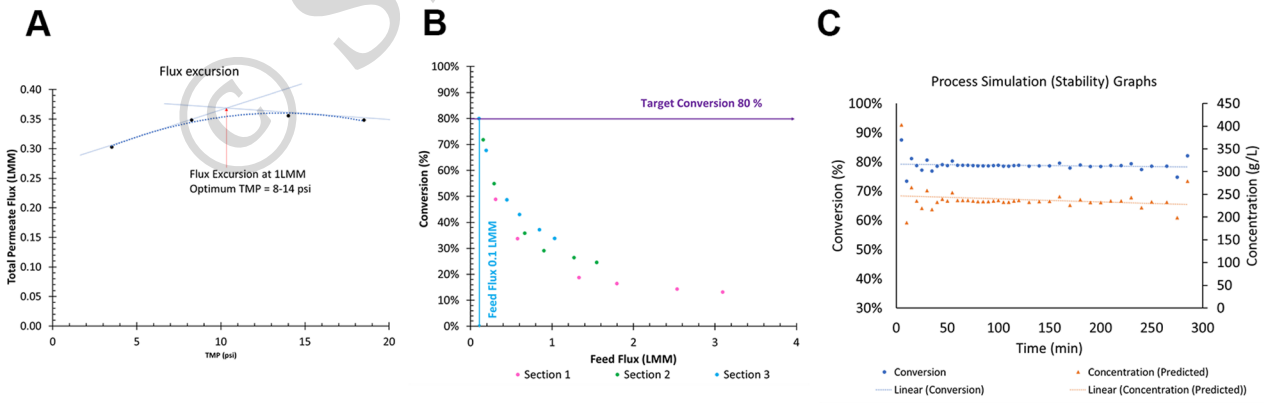


Figure 4 - Single-pass tangential flow filtration (SPTFF) optimisation, concentration, and simulation

(A) Flux excursion to define optimal TMP at 11-12 pound per square inch (psi). (B) Feed flux identification with three sections of membrane cassettes at target 80% conversion at 0.1 LMM feed flux. (C) Simulation run over 300 minutes (min) maintained a steady conversion rate to reach 200 mg/mL target concentration. LMM: feed flow rate in L per min per total square meters of membrane area; TMP: transmembrane pressure.

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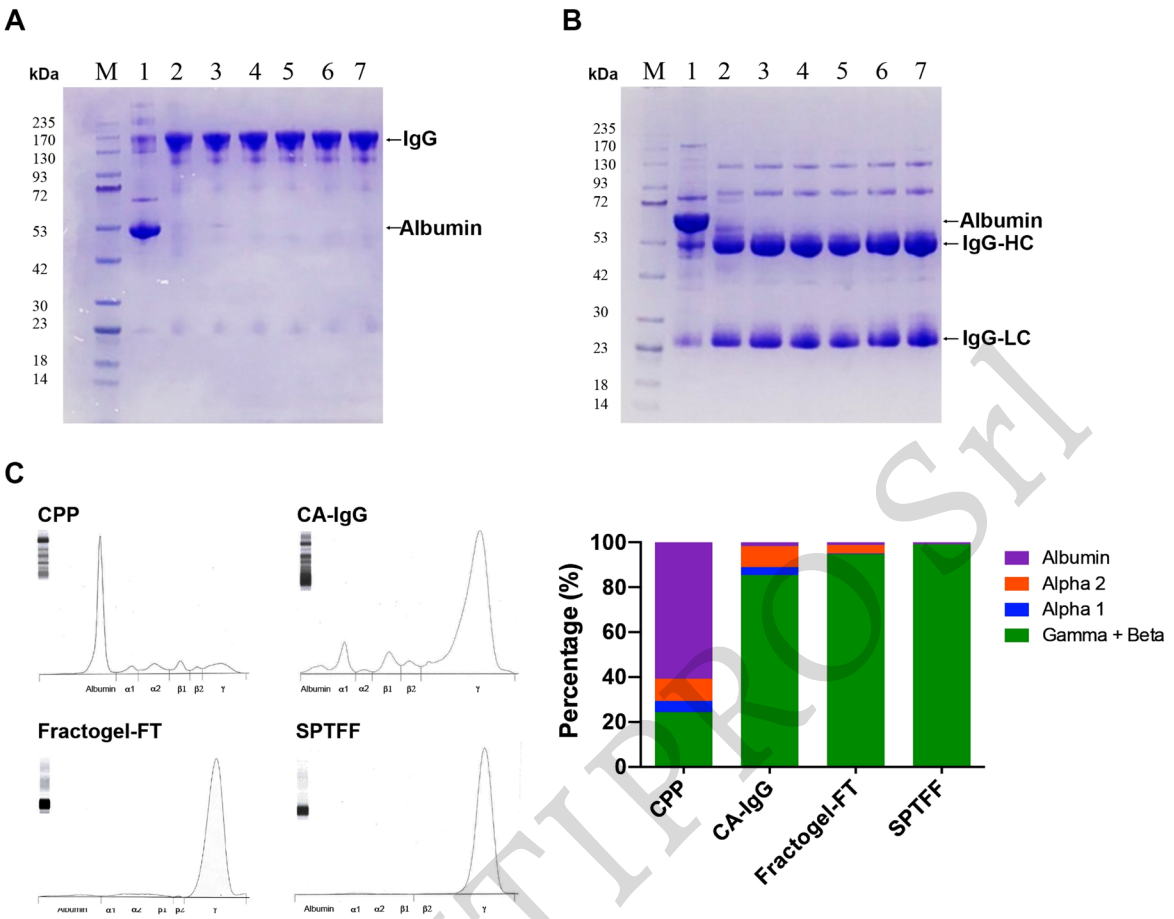


Figure 5 - SDS-PAGE under non-reducing (A) and reducing (B) conditions of fractions associated with each purifying procedure

M: protein marker (14-245 kDa); 1: CPP, 2: CA-IgG, 3: Fractogel® TMAE-FT, 4: Anti-A-FT, 5: Anti-B-FT, 6: C18-FT, 7: SPTFF. Proteins of the IgG fractions along the purification process are separated by zone electrophoresis (C). CPP: cryo-poor plasma; CA-IgG: caprylic acid-treated immunoglobulin; Fractogel® TMAE: flow through of fractions passing through Fractogel® TMAE; SPTFF: single-pass tangential flow filtration.

Table I - *Thrombin generation assay (TGA) conducted on IgG fractions over the various stages of purification*

In-process IgG fractions	RC high				
	Lag phase (min)	Thrombin (nM)	Time to peak (min)	Velocity index	AUC
CPP	4.75±0.50	52.16±14.73	27.25±19.92	6.98±6.93	954.20±325.20
CA-IgG	4.00±0.00	27.20 ±5.92	44.50±0.58	0.67±0.15	291.24±155.68
Fractogel® TMAE	0	0	0	0	0
Anti-A	0	0	0	0	0
Anti-B	0	0	0	0	0
C18	0	0	0	0	0
SPTFF	0	0	0	0	0

AUC: area under the curve; CPP: cryoprecipitate-poor plasma; IgG: immunoglobulin G; SPTFF: single-pass tangential flow filtration; min: minutes.

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Assessment of *in vitro* thrombogenicity

FXI was not detectable by FXIa assay after caprylic acid treatment. Thrombin generation assay consistently showed the lack of thrombin generation activity after purification by Fractogel® TMAE in both the small-scale and in the pilot experiments, with a removal of thrombin generation factors during Fractogel® TMAE (**Table I**).

DISCUSSION

Human plasma IgG have multiple clinical indications in the treatment of immunological and infectious disorders[9,18,41]. Due to persistent shortages, easy-to-implement "generic" technologies for IgG purification from various starting materials, capable of removing unwanted contaminants and to ensure a virus safety margin should be made available. As highlighted in a recent WHO Guidance document, scalable technologies could, in a stepwise approach, serve the needs of emerging countries where plasma is prepared but currently discarded because of lack of local fractionation infrastructure or because the volume available yearly is not sufficient to meet requirements for contract fractionation[42,43].

We thus evaluated several steps for IgG fractionation to facilitate and speed-up product development and implementation. The recognised features of a modern IgG purification process are its capacity to provide an IgG preparation of high-purity, depleted of IgA and isoagglutinins, and devoid of thrombogenic factors[20,44]. In addition, the resulting IgG preparation should be amenable to concentration to 20% (200 mg/mL) to meet the growing clinical demand of primary immunodeficient patients for SC administration at home[5,45].

To develop such a methodology, as feed material for experimental purposes, we used a "crude" immunoglobulin fraction obtained by caprylic acid precipitation of CPP, as previously described[38-40]. Selecting this fraction was based on the following rationale: 1) it removed the need for a cold ethanol Cohn fractionation process and therefore could be readily implemented at small and pilot scales[38,40,46,47]; 2) it represented a challenging worst-case scenario for process development compared to other plasma intermediates such as Cohn supernatant III or precipitate II, as its IgG purity is only approximately 90% with a level of contamination by IgA and IgM similar to that of the starting plasma; and 3) caprylic acid, which has long been used in the manufacture of horse plasma-derived antivenom IgG[48], is used in the purification and virus inactivation of licensed human IgG[31,32,49,50] and preparation of mini-pool or convalescent IgG[46,51]. It should be borne in mind, however, that applying a caprylic acid treatment directly on CPP, although convenient experimentally to obtain a crude Ig fraction model, is not optimal for industrial human plasma fractionation of standard plasma as albumin is lost in the precipitate and hard to recover.

The developed IgG processing steps that were evaluated use a flow-through mode for optimal performance and ease of use. In particular, we selected anion-exchange to adsorb IgA as it is more suitable than bind-elute approaches of IgG by cation exchanger for process implementation. Although cation exchangers are convenient to remove virus inactivating agents in the flow-through subsequent to a S/D treatment[38,40,52], one drawback is a limited binding capacity of IgG, which affects productivity. Using Fractogel® TMAE(M), up to 225 mg of the IgG solution, including approximately 40.8 mg of IgA and 5.86 mg of IgM, could be injected per mL, leading to a consistent reduction of 45.64% and 38.6% in the IgA/IgG and IgM/IgG ratio over 201 cycles to assess robustness. At a lower loading level, down to 87.63 mg IgG, a good removal (96.89%) of IgM could also be achieved. Under optimal conditions, the removal of IgA was as high as 96.16%[53]. Removal of IgA is thought to limit the risk of anaphylactic reactions in IgA-deficient patients who have anti-IgA antibodies[54]. The optimised chromatographic conditions provided satisfactory preservation of the IgG sub-class distribution, although sub-class 4 is more susceptible to bind to an anion exchanger and therefore to be removed. The reproducibility of the flow-through IgG purification process was confirmed by a series of 201 injection, washing and sanitisation cycles, with no significant change in IgG purity, 94.22% recovery, preservation of subclass distribution, or 96.16% IgA or 96.89% IgM removal. The recovery rate of IgG, IgA and IgM was calculated by comparing the Ig content of the collected flow-through with the Ig content in the loaded CA-IgG.

For anti-A and anti-B removal, we used newly developed affinity resins intended for dedicated capture of blood group isoagglutinins A and B[55]. The anti-A removal step was performed first, followed by the anti-B, although

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the chromatographic sequence could likely be reversed. Alternatively, mixing the two resins in equal or different amounts packed in the same column for process optimisation should be feasible as both resins were operated under the same chromatographic conditions. Both polyvinylether-based resins, functionalised by trisaccharide blood group antigens A and B, respectively, successfully depleted the isoagglutinins from the Fractogel® TMAE purified IgG. A recent clinical report indicated that isoagglutinin depletion can decrease the occurrence of haemolytic reactions by 90%, particularly in patients treated with high-dose IgG[34].

The S/D treatment was applied on an essentially pure IgG fraction obtained after Fractogel® TMAE and the two Eshmuno® P columns. We combined 0.3% TnBP and 1% Triton X-100 to provide a step capable of inactivating the enveloped viruses, based on current practice in the industry[21,39]. Such S/D treatment can be conducted in a specially designed single-use Pureflex™ coated bag under conditions leading to robust virus inactivation and low leachable levels[39]. The silica-based packing material exhibited a binding capacity for the S/D agents at a ratio of 0.63 g of C18 for up to 17 mL of S/D-IgG similar to or even higher than that identified for another C18 material[56] found to have a binding capacity of >140 mg of TnBP per g, and >70 mg of Triton X-45 per g. At pilot scale, a "conservative" volume of 973 mL of the S/D-containing IgG solution could be chromatographed on 160 mL of C18 sorbent (corresponding to 6 mL of SD-treated IgG solution/mL of sorbent) at a residence time of 5 min, with undetectable TnBP (<1 ppm) and Triton X-100 (<2 ppm) in the flow-through. Furthermore, the S/D-treated IgG was non-toxic to 3T3 cells, confirming the removal of the virus inactivating agents. Development of this S/D procedure should consider the restrictions on the use of Triton X-100 in some countries, and the expected availability of a substitute detergent[57]. As the S/D treatment inactivates only the lipid-enveloped viruses, an additional in-process virus reduction step, such as nanofiltration[58] and/or low pH incubation[25,59], should definitely be considered for large-scale industrial IgG production. Regardless of the combination of virus reduction steps selected in a fractionation process, each of them should be carefully validated following current regulatory requirements[60]. The content in FXI/FXIa has been considered to be a contributing factor[61] of thrombo-embolic effects of IVIG preparation[62,63]. Our data show that the final IgG had undetectable FXI/FXIa, also confirming the capacity of the caprylic acid treatment to remove or inactivate FXIa[38]. In addition, the purified IgG did not exhibit any thrombin generation activity as the Fractogel® TMAE was consistently found to remove the procoagulant factors leading to thrombin generation. It is important to keep in mind that IgG manufacturers implementing such a process on their IgG intermediate would also be required to perform additional quality control tests, not conducted here, for process validation or end-product quality control, such as anti-complementary activity, prekallicrein activator, size-distribution, as specified in various pharmacopoeias[20], as well as stability studies of the formulated end-product.

In conclusion, it is possible to implement process steps based on Fractogel® TMAE, Eshmuno® P anti-A, and Eshmuno® P anti-B chromatography to obtain an isoagglutinin-depleted IgG fraction with well-maintained IgG sub-class distribution and depleted IgA and thrombogenic activity. This fraction can be S/D-treated and processed by C18 LiChroprep® RP-18, and concentrated to 20%. These process steps used in a flow-through mode can be reliably monitored by controlling key process parameters, and should be readily applicable to various IgG intermediates generated by plasma fractionation.

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AUTORSHIP CONTRIBUTIONS

JHCC and YWW contributed equally to this work.

JHCC and TB conceived and designed the experiments and

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the set of quality control tests. CYW, SSJW, YWW, and CLH performed the experiments. All Authors reviewed the data. TB, YWW, and JHCC wrote the manuscript.

CONFLICT OF INTEREST

JHCC, SSJW, KWC, LXL, XC and BW are employees of Merck. YWW, CYW, CLH and TB declare no conflict of interest.

REFERENCES

1. Farrugia A, Robert P. Plasma protein therapies: current and future perspectives. Best Pract Res Clin Haematol 2006; **19**: 243-58.
2. Toubi E, Etzioni A. Intravenous immunoglobulin in immunodeficiency states: state of the art. Clin Rev Allergy Immunol 2005; **29**: 167-72.
3. Bonagura VR. Using intravenous immunoglobulin (IVIG) to treat patients with primary immune deficiency disease. J Clin Immunol 2013; **33** (Suppl 2): S90-4.
4. Simoens S. Pharmacoeconomics of immunoglobulins in primary immunodeficiency. Expert review of pharmacoeconomics & outcomes research 2009; **9**: 375-86.
5. Mallick R, Henderson T, Lahue BJ, et al. Subcutaneous immunoglobulin in primary immunodeficiency - impact of training and infusion characteristics on patient-reported outcomes. Bmc Immunology 2020; **21**: 1-15.
6. Prevot J, Jolles S. Global immunoglobulin supply: steaming towards the iceberg? Curr Opin Allergy Clin Immunol 2020; **20**: 557-64.
7. Monleón Bonet C, Waser N, Tzivelekis S, et al. A systematic literature review of the effects of immunoglobulin replacement therapy on the burden of secondary immunodeficiency diseases associated with hematological malignancies and stem cell transplants. Expert Rev Clin Immunol 2020; **16**: 911-21.
8. Kivity S, Katz U, Daniel N, et al. Evidence for the use of intravenous immunoglobulins-a review of the literature. Clin Rev Allergy Immunol 2010; **38**: 201-69.
9. Negi VS, Elluru S, Siberil S, et al. Intravenous immunoglobulin: an update on the clinical use and mechanisms of action. J Clin Immunol 2007; **27**: 233-45.
10. Perez EE, Orange JS, Bonilla F, et al. Update on the use of immunoglobulin in human disease: A review of evidence. J Allergy Clin Immunol 2017; **139**: S1-S46.
11. Mair-Jenkins J, Saavedra-Campos M, Baillie JK, et al. The effectiveness of convalescent plasma and hyperimmune immunoglobulin for the treatment of severe acute respiratory infections of viral etiology: a systematic review and exploratory meta-analysis. J Infect Dis 2015; **211**: 80-90.
12. Zhang Z, Xie YW, Hong J, et al. Purification of severe acute respiratory syndrome hyperimmune globulins for intravenous injection from convalescent plasma. Transfusion 2005; **45**: 1160-4.
13. Burnouf T, Seghatchian J. Ebola virus convalescent blood products: where we are now and where we may need to go. Transfus Apher Sci 2014; **51**: 120-5.
14. Piechotta V, Chai KL, Valk SJ, et al. Convalescent plasma or hyperimmune immunoglobulin for people with COVID-19: a living systematic review. Cochrane Database Syst Rev 2020; **7**: CD013600.
15. WHO Model Lists of Essential Medicines WHO Model List of Essential Medicines: 21st list 2019. Geneva: World Health Organization; 2019 Available at: https://apps.who.int/iris/handle/10665/325771, 2019. Accessed on 19/06/2021.
16. Norris PAA, Kaur G, Lazarus AH. New insights into IVIg mechanisms and alternatives in autoimmune and inflammatory diseases. Curr Opin Hematol 2020; **27**: 392-8.
17. Spath PJ, Schneider C, von Gunten S. Clinical Use and Therapeutic Potential of IVIG/SCIG, Plasma-Derived IgA or IgM, and Other Alternative Immunoglobulin Preparations. Arch Immunol Ther Exp (Warsz) 2017; **65**: 215-31.
18. Shemer A, Kivity S, Shoenfeld Y. Clinical indications for intravenous immunoglobulin utilization in a tertiary medical center: a 9-year retrospective study. Transfusion 2018; **58**: 430-8.
19. Bousfiha AA, Duff C, Hsieh E. Ensuring Access to Immunoglobulin Therapies for People with Primary Immunodeficiency: A Need to Improve Individuals' Quality of Life and the Sustainability of Health-care Systems. Front Immunol 2017; **8**: 1165.
20. Radosevich M, Burnouf T. Intravenous immunoglobulin G: trends in production methods, quality control and quality assurance. Vox Sang 2010; **98**: 12-28.
21. Dichtelmuller HO, Biesert L, Fabbrizzi F, et al. Robustness of solvent/detergent treatment of plasma derivatives: a data collection from Plasma Protein Therapeutics Association member companies. Transfusion 2009; **49**: 1931-43.
22. Burnouf-Radosevich M, Appourchaux P, Huart JJ, et al. Nanofiltration, a new specific virus elimination method applied to high-purity factor IX and factor XI concentrates. Vox Sang 1994; **67**: 132-8.
23. Van Holten RW, Autenrieth S, Boose JA, et al. Removal of prion challenge from an immune globulin preparation by use of a size-exclusion filter. Transfusion 2002; **42**: 999-1004.
24. Burnouf T. Modern plasma fractionation. Transfus Med Rev 2007; **21**: 101-17.
25. Burnouf T, Radosevich M. Reducing the risk of infection from plasma products: Specific preventative strategies. Blood Rev 2000; **14**: 94-110.
26. Alving BM, Tankersley DL, Mason BL, et al. Contact-activated factors: contaminants of immunoglobulins preparations with coagulant and vasoactive properties. J Lab Clin Med 1980; **96**: 334-46.
27. Etscheid M, Breitner-Ruddock S, Gross S, et al. Identification of kallikrein and FXIa as impurities in therapeutic immunoglobulins: implications for the safety and control of intravenous blood products. Vox Sang 2012; **102**: 40-6.
28. Wolberg AS, Kon RH, Monroe DM, et al. Coagulation factor XI is a contaminant in intravenous immunoglobulin preparations. Am J Hematol 2000; **65**: 30-4.
29. Daniel GW, Menis M, Sridhar G, et al. Immune globulins and thrombotic adverse events as recorded in a large administrative database in 2008 through 2010. Transfusion 2012; **52**: 2113-21.
30. Cunningham-Rundles C, Zhou Z, Mankarious S, et al. Long-term use of IgA-depleted intravenous immunoglobulin in immunodeficient subjects with anti-IgA antibodies. J Clin Immunol 1993; **13**: 272-8.
31. Goussen C, Simoneau S, Berend S, et al. Biological Safety of a Highly Purified 10% Liquid Intravenous Immunoglobulin Preparation from Human Plasma. BioDrugs 2017; **31**: 251-61.
32. Lebing W, Remington KM, Schreiner C, et al. Properties of a new intravenous immunoglobulin (IGIV-C, 10%) produced by virus inactivation with caprylate and column chromatography. Vox Sang 2003; **84**: 193-201.
33. Gerber S, Gaida A, Spiegl N, et al. Reduction of Isoagglutinin in Intravenous Immunoglobulin (IVIG) Using Blood Group A- and B-Specific Immunoaffinity Chromatography: Industry-Scale Assessment. BioDrugs 2016; **30**: 441-51.
34. Wallenhorst C, Patel A, Shebl A, et al. Anti-A/B isoagglutinin reduction in an intravenous immunoglobulin product and risk of hemolytic anemia: a hospital-based cohort study. Transfusion 2020; **60**: 1381-90.
35. Yazdanbakhsh K, Zhong H. IVIg and hemolytic anemia risk: dump the anti-A/B already! Transfusion 2020; **60**: 1337-9.
36. Alonso W, Vandeberg P, Lang J, et al. Immune globulin subcutaneous, human 20% solution (Xembify(R)), a new high concentration immunoglobulin product for subcutaneous administration. Biologicals 2020; **64**: 34-40.
37. Espanol T, Prevot J, Drabwell J, et al. Improving current immunoglobulin therapy for patients with primary immunodeficiency: quality of life and views on treatment. Patient preference and adherence 2014; **8**: 621.
38. Wu YW, Champagne J, Toueille M, et al. Dedicated removal of immunoglobulin (Ig)A, IgM, and Factor (F)XI/activated FXI from human plasma IgG. Transfusion 2014; **54**: 169-78.
39. Hsieh YT, Mullin L, Greenhalgh P, et al. Single-use technology for solvent/detergent virus inactivation of industrial plasma products. Transfusion 2016; **56**: 1384-93.
40. Weng YJ, Husebekk A, Skogen B, et al. Anti-Human Platelet Antigen-1a Immunoglobulin G Preparation Intended to Prevent Fetal and Neonatal Alloimmune Thrombocytopenia. PLoS One 2016; **11**: e0162973.

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41. Garraud O. Injectable immunoglobulins, immunodeficiency and off-label clinical trials, source plasma and ethical concerns and debates. Transfus Apher Sci 2019; **58**: 529-30.
42. Burnouf T, Faber JC, Radosevic M, et al. Plasma fractionation in countries with limited infrastructure and low-/medium income: how to move forward? Transfus Apher Sci 2020; **59**: 102715.
43. WHO White Paper. Increasing supplies of plasma-derived medicinal products in low- and middle-income countries through fractionation of domestic plasma (in press) 2021.
44. Hooper JA. Intravenous immunoglobulins: evolution of commercial IVIG preparations. Immunol Allergy Clin North Am 2008; **28**: 765-78, viii.
45. Chapel HM, Spickett GP, Ericson D, et al. The comparison of the efficacy and safety of intravenous versus subcutaneous immunoglobulin replacement therapy. Journal of Clinical Immunology 2000; **20**: 94-100.
46. El-Ekiaby M, Vargas M, Sayed M, et al. Minipool caprylic acid fractionation of plasma using disposable equipment: a practical method to enhance immunoglobulin supply in developing countries. PLoS Negl Trop Dis 2015; **9**: e0003501.
47. Morais V, Berasain P, Massaldi H. Immunoglobulin purification by caprylic acid. Methods Mol Biol 2014; **1129**: 137-43.
48. Rojas G, Jimenez JM, Gutierrez JM. Caprylic acid fractionation of hyperimmune horse plasma: description of a simple procedure for antivenom production. Toxicon 1994; **32**: 351-63.
49. Parkkinen J, Rahola A, von Bonsdorff L, et al. A modified caprylic acid method for manufacturing immunoglobulin G from human plasma with high yield and efficient virus clearance. Vox Sang 2006; **90**: 97-104.
50. Dichtelmuller H, Rudnick D, Kloft M. Inactivation of lipid enveloped viruses by octanoic acid treatment of immunoglobulin solution. Biologicals 2002; **30**: 135-42.
51. Ali S, Uddin SM, Ali A, et al. Production of hyperimmune anti-SARS-CoV-2 intravenous immunoglobulin from pooled COVID-19 convalescent plasma. Immunotherapy 2021; **13**: 397-407.
52. Poelsler G, Berting A, Kindermann J, et al. A new liquid intravenous immunoglobulin with three dedicated virus reduction steps: virus and prion reduction capacity. Vox Sang 2008; **94**: 184-92.
53. Lemm G. Composition and properties of IVIg preparations that affect tolerability and therapeutic efficacy. Neurology 2002; **59**: S28-32.
54. de Albuquerque Campos R, Sato MN, da Silva Duarte AJ. IgG anti-IgA subclasses in common variable immunodeficiency and association with severe adverse reactions to intravenous immunoglobulin therapy. J Clin Immunol 2000; **20**: 77-82.
55. Rahane S, Turiano M, Stone M, et al. Eshmuno (R) P anti-A and anti-B: Affinity resins for removal of anti-A and anti-B isoagglutinins. Abstracts of papers of the American Chemical Society: Amer Chemical Soc 1155 16th st, NW, Washington, DC 20036 USA, 2018.
56. Burnouf T, Sayed MA, Radosevich M, et al. Comparative removal of solvent and detergent viral inactivating agents from human intravenous immunoglobulin G preparations using SDR HyperD and C18 sorbents. Anal Biochem 2009; **389**: 69-73.
57. Luo W, Hickman D, Keykhosravani M, et al. Identification and characterization of a Triton X-100 replacement for virus inactivation. Biotechnol Prog 2020; **36**: e3036.
58. Roth NJ, Dichtelmuller HO, Fabbrizzi F, et al. Nanofiltration as a robust method contributing to viral safety of plasma-derived therapeutics: 20 years' experience of the plasma protein manufacturers. Transfusion 2020; **60**: 2661-74.
59. Roberts PL, Dunkerley C, Walker C. Virus reduction in an intravenous immunoglobulin by solvent/detergent treatment, ion-exchange chromatography and terminal low pH incubation. Biologicals 2012; **40**: 345-52.
60. CPMP. Note for guidance on plasma-derived medicinal products. CPMP/BWP/269/95 rev. 3. Available at: https://www.ema.europa.eu/en/documents/scientific-guideline/note-guidance-plasma-derived-medicinal-products_en.pdf. London, 2001. Accessed on 19/06/2021.
61. Roemisch JR, Kaar W, Zoechling A, et al. Identification of activated FXI as the major biochemical root cause in IVIG batches associated with thromboembolic events. Analytical and experimental approaches resulting in corrective and preventive measures implemented into the Octagam® manufacturing process. WebmedCentral Immunotherapy 2011; **2**: WMC002002.
62. FDA US. Octagam (Immune Globulin Intravenous (human)) 5% Liquid Preparation: Market Withdrawal - Risk of Thromboembolic Events [monograph on the internet]. Bethesda; 2010. Available from: http://www.fda.gov/Safety/MedWatch/SafetyInformation/SafetyAlertsforHumanMedicalProducts/ucm223968.htm. Accessed on 19/06/2021.
63. EMA. European Medicines Agency recommends suspension of Octagam in all EU member states. CHMP recommendation based on risk of thromboembolic reactions. EMA/CHMP/591722/2010. 24 September 2010. [monograph on the internet]. London. Available at: http://www.mhra.gov.uk/Safetyinformation/Safetywarningsalertsandrecalls/Safetywarningsandmessagesformedicines/CON094141. Accessed on 19/06/2021.

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