A new manufacturing process to remove thrombogenic factors (II, VII, IX, X, and XI) from intravenous immunoglobulin gamma preparations

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Coagulation factors (II, VII, IX, X, and particularly XIa) remaining in high concentrations in intravenous immunoglobulin (IVIG) preparations can form thrombi, causing thromboembolic events, and in serious cases, result in death. Therefore, manufacturers of biological products must investigate the ability of their production processes to remove procoagulant activities. Previously, we were able to remove coagulation factors II, VII, IX, and X from our IVIG preparation through ethanol precipitation, but factor XIa, which plays an important role in thrombosis, remained in the intermediate products. Here, we used a chromatographic process using a new resin that binds with high capacity to IgG and removes procoagulant activities. The procoagulant activities were reduced to low levels as determined by the thrombin generation assay: <1.56 mIU/mL, chromogenic FXIa assay: <0.16 mIU/mL, non-activated partial thromboplastin time (NaPTT): >250 s, FXI/FXIa ELISA: <0.31 ng/mL. Even after spiking with FXIa at a concentration 32.5 times higher than the concentration in normal specimens, the procoagulant activities were below the detection limit (<0.31 ng/mL). These results demonstrate the ability of our manufacturing process to remove procoagulant activities to below the detection limit (except by NaPTT), suggesting a reduced risk of thromboembolic events that may be potentially caused by our IVIG preparation.

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1. Introduction

Intravenous immunoglobulin G (IVIG) has been used for treating various immunological deficiencies and autoimmune disorders. Different manufacturers of IVIG use different manufacturing processes, resulting in differences in the final product composition [1–5].

In general, treatments involving IVIG use purified IgG from human plasma, which ensures high safety of the preparations. Such treatments have been widely used until now, except in rare cases, such as in patients with IgA deficiency [6]. However, recently, thromboembolic events were found to have occurred in a patient who had received a high dose of IVIG. Thromboembolic events are induced when the coagulation factor XIa (FXIa) remaining in the IVIG preparation (as a result of imperfect removal in the manufacturing process) activates the coagulation cascade in the blood of the recipient [7,8].

Consequently, the revised European pharmacopoeia now recommends that manufacturers include a quality check to ensure the removal of thrombosis-generating agents (coagulation factors and their zymogens) in IVIG manufacturing processes or submit evidences to indicate that the product does not induce zymogen activation [9].

Typically, the ethanol precipitation method developed in the 1940s is used to separate IgG from plasma [10,11]. However, it is difficult to completely isolate and purify IgG from the FXIa only through ethanol precipitation due to the high isoelectric point (pI 8.9–9.1) of FXIa [12]. Therefore, an additional purification process is...
required to remove any remaining FXIa.

Effective removal of FXIa is a very important aspect in the manufacture of safe IVIG preparations. During the manufacturing process, this step is conducted using various methods such as caprylic acid precipitation and membrane chromatography [13, 14]. However, in the case of preparations with high concentrations of IVIG (e.g., blood preparations), the use of a membrane filter reduces the overall yield or increases the cost of materials required for the process; thus, it is difficult to introduce this step into the manufacturing process. It is therefore necessary to develop a manufacturing process that can provide sufficient yield and can maximize the safety of the preparations without increasing the manufacturing time.

GC5101B is a high-purity pharmaceutical IVIG preparation manufactured through cold ethanol fractionation and chromatography. In this study, we developed a new process for reducing the content of FXIa in IVIG preparations to levels below the detection limits to ensure the safety of GC5101B. Furthermore, since blood products are generally derived from plasma from over 1000 donors, there are differences in protein quantity and composition depending on the country or state of the donor. Therefore, we further spiked our samples with additional coagulation factor Xla, in amounts exceeding any variability that may be caused due to sample differences, and were able to still successfully remove Xla, thus verifying the robustness of our manufacturing process.

2. Materials and methods

2.1. Materials

The intermediate and final purification products of GC5101B from the original manufacturing process were provided by Green Cross Corp. (Korea), and the standard coagulation-factor materials for the spiking study were purchased from HTI (Hematologic Technologies Inc., USA). For the thrombin generation assay (TGA), the WHO reference reagent NIBSC (1st international reference reagent for activated blood coagulation factor XI (FXIa), Human NIBSC code: 11/236) was used; for the non-activated prothrombin time test (NaPTT) assay, NIBSC's factor XIa was used. For the chromogenic FXIa assay, a biophen factor Xla kit (Chromogenix, UK) was used.

2.2. Study design

During the manufacturing process, cryo-poor pooled plasma was used as a starting material and a paste containing Fraction I + II + III, a supernatant containing Fraction I + III, and a Fraction II paste was produced through cold ethanol fractionation. The starting material for the manufacturing process for GC5101B was the Fraction II paste obtained by cold ethanol precipitation. This Fraction II paste was dissolved into 4-fold volume of 0.6% sodium chloride solution at below 10°C. Next, the pH was adjusted to 5.0, which was followed by clarifying depth filtration (nominal size: 0.1 μm). 1 M sodium acetate was added to the diafiltrated IgG solution to a final concentration of 5.0 mM; the pH was then adjusted to 6.0 and the solution was subjected to AEX chromatography. The unbound fraction was collected and its pH was adjusted to 5.0 before subjecting it to the solvent/detergent virus inactivation process. Tri(n-butyl)-phosphate (TNBP) and Polysorbate 80 (Tween 80) were added at concentrations of 0.3% and 1.0%, respectively, and the mixture was then incubated at 25°C for 8 h. To bring the final concentration to 20 mM, 1 M sodium acetate was added to the filtrate, and the filtrate was subjected to CEX chromatography to absorb IgG. Another clarifying depth filtration was then performed at the same pH just before CEX chromatography. After CEX chromatography, the filtrate was washed with equilibrium buffer (20 mM sodium acetate, pH 5.0) and then eluted with elution buffer (20 mM sodium acetate with 0.5 M sodium chloride, pH 4.5) to acquire IgG. The salt content was eliminated by Ultra/Dia-filtration. A nanofilter (Ultipur®VF DV 20, Pall Lifescience, Switzerland) was used to eliminate viruses, and stabilizers were applied. Finally, after sterile filtration, the finished product was obtained. We then determined the procoagulant activities from each of the intermediate and final products.

2.3. Detection of FXI/FXIIa

FXII/FXIIa content in the intermediate products of GC5101B—Fraction I + II + III Paste, Fraction I + III supernatant, and Fraction II Paste—was analyzed using western blot and ELISA, and its procoagulant activity was analyzed using TGA. The procoagulant activity of the final product was measured using TGA, chromogenic FXIa assay, and NaPTT assay. Each method was verified based on ICH Guideline Q2 (R1) [15] and was used in the analysis of the intermediate and final products.

2.4. TGA

TGA was conducted in accordance with “CBER Ig-Thrombin Generation Test Protocol (automated version)” [16]. There are two methods for the determination of the potential risk of thromboembolic events: Wessler test (in vivo) and TGA (in vitro). TGA is known to be the most sensitive and reliable method for detecting thrombogenic agents in plasma-derived preparations. To measure FXIa contents, the test sample was treated with a mixture of 50 μL Factor XI (FXI)-deficient plasma (Hematologic Technologies Inc., USA), 2.5 μL tissue factor ((Dade Innovin®, USA), 2.5 μL phospholipid (Rossix, Sweden) and 8.75 μL of CaCl₂ (Sigma Aldrich, USA). Activated thrombin in the test samples were measured by analyzing the release of a fluorophore from a fluorogenic substrate (Z-Gly-Gly-Arg-AMC, Bachem, Switzerland) with a kinetic fluorescent reader (Infinite F-500, Tecan, Switzerland; excitation wavelength: 380 nm, emission wavelength: 430 nm) at 37°C. This value was applied to the standard curve constructed with fluorescence measurements obtained for standard product (FXIa, NIBSC 11/236). Undiluted IgG specimen, FXIa standard (FXIa, NIBSC 11/236) and IVIG from normal production batches were used as controls. IVIG samples, from a high or low procoagulant Ig lot, were selected to measure reproducibility.

2.5. NaPTT assay

The NaPTT assay was performed to determine the coagulation time as a means to measure the activities of coagulant factors. The NaPTT assay was conducted according to Ph. Eur. 2.6.22 [17]. The Fraction I + II + III paste was dissolved in sterile water (Water for Injection, manufacturing site) at a 1:5 ratio for 5–7 h, followed by filtration using a syringe filter. The Fraction II paste was dissolved in 0.6% sodium chloride at a 1:4 ratio for 1 h. Cryo-poor plasma, dissolved Fraction I + II + III Paste, Fraction I + III supernatant, and dissolved Fraction II Paste were further diluted 10 times with Tris-albumin buffer. Citrated plasma was used as a control. A series of polystyrene tubes were placed in a water-bath at 37°C and 0.1 mL of platelet-poor plasma substitute R was added to each tube. 0.1 mL of the diluted samples or 0.1 mL of the buffer (control tube) was added to the tubes. Then, 0.1 mL of a 3.7 g/L solution of calcium chloride R (previously warmed to 37°C) was added immediately to all the tubes. Clotting time was measured using a manual coagulator (Sigma KC4 coagulator, Amelung, Germany) within 30 min of adding the original dilution as the time that elapses between the
addition of the calcium chloride solution and the formation of a clot. This test is not valid unless the coagulation time measured for the control tube is 200 s–300 s. We also measured the clotting time using a ACL TOP 500 (Instrumentation Laboratory, USA).

2.6. Chromogenic FXa assay

FXa activity was measured using the ROX FACTOR Xla test kit (Rossix, Sweden). FIX, FVIII, and calcium chloride were added to the specimen to activate FIX for conversion to FXa. When the conversion of FX to FXa was induced by activated FXa, the activated FXa hydrolyzed the substrate (Z-D-Arg-Gly-Arg-pNA) and produced a chromogenic product (p-nitroalanine), which was measured at a wavelength of 405 nm (reference wavelength: 490 nm). FXa activity was then measured by comparison with that of the standard solution (biophen factor XIa kit, Chromogenix, UK).

2.7. Immunoblotting

For immunoblotting, each sample was subjected to SDS-PAGE and then electroblotted onto a polyvinylidene difluoride (PVDF) membrane by semi-dry transfer (Bio-Rad, USA). The membrane was then blocked for 1 h and incubated overnight at 4 °C with the indicated primary antibody (Anti-human Factor XI primary polyclonal antibody, Innovative Research, USA), followed by incubation with horseradish peroxidase-conjugated secondary antibodies for 1 h (Innovative Research, USA). The signals were then detected using chemiluminescence reagents (Intron, USA).

3. Results

When each intermediate product was analyzed using ELISA, the percentages of FXa remaining were 74.4%, 5.8%, and 5.2% in the Fraction I + II + III paste, Fraction I + III supernatant, and Fraction II paste, respectively. The comparison of these percentages to those in the cryo-poor pooled plasma is shown in Fig. 1. The results show that 5.2% FXa was remaining, which indicates that cold ethanol fractionation alone could not remove FXa completely.

The procoagulant activity of the intermediate products in each process was determined using ELISA (for the amount of FXI/FXIIa), TGA, chromogenic FXa assay, Prekallikrein activator (PKA) test, kallikrein activity measurement, and NaPTT assay. The results indicate that cold ethanol fractionation process removed most of the procoagulant activity, but not all of it (Fig. 2). As shown in Fig. 2, cryo-poor plasma showed increased procoagulant activity by the TGA, chromogenic FXa assay, PKA test, Kallikrein activity test, and NaPTT; the FXI/FXIIa content measured by ELISA, however, showed a contrasting result.

We confirmed that coagulation factor VII was removed using supernatant removal from the Fraction I + II + III, and that FII, FIX, and FX were removed using precipitation removal from Fraction I + III. However, FXI/FXIIa was not removed completely, leaving some amount in the Fraction II paste (Fig. 3).

We then attempted to improve the product safety of GC5101B by including anion exchange and cation exchange chromatographic processes during the manufacturing for the removal of plasma-derived impurities and procoagulant activity remaining in the Fraction II paste and by adding the virus inactivation (S/D treatment) and virus removal (nano-filtration) processes (Fig. 4).

Anion exchange chromatography was effective in removing plasma-derived impurities and PKA, but could not remove FXa (Fraction II paste: 13.0 ng/mL, pre-CEX chromatography: 10.3 ng/mL). Coagulation factor Xla was then removed using CEX chromatography. The ceramic-based resin (CM Ceramic Hyper D Sorbent, Pall Life Sciences) used in CEX chromatography has 1.5 times higher binding capacity compared to commonly used resins in protein purification. This allowed the column to be more compact to efficiently reduce the amount of buffer required and the time required for the production process (binding capacity: 100.0 g/L of resin or higher). In addition, with a loading of pH 5.0, the elution pH was adjusted to 4.5 and Ultra/Diafiltration was conducted at the same time as IgG elution to eliminate the risk of reversible polymerization due to salt (Table 1). Samples were analyzed by western blot before and after CEX chromatography, and the results showed that FXI/FXIIa was reduced to an undetectable level (Fig. 3). FXa absorbed with IgG was not eluted under high salt concentrations (2.0 M) and was removed in 6.0 M guanidine hydrochloride CIP. Considering that the elution salt level is 500 mM in CEX chromatography, there is no risk of introducing FXIIa after CEX chromatography process.

Since IVIG uses various types of plasma samples as the starting material, there can be a variation in the content of FXa. Therefore, a spiking study was conducted to evaluate the removal rate of FXa during CEX chromatography. In general, the concentration of FXa in plasma is 4–6 μg/mL [8,18]. The manufacturing process for GC5101B could remove approximately 95% of the FXa through cold ethanol fractionation. However, since the amount of FXa varies depending on the plasma type, the effectiveness of the removal process for various plasma types needs to be verified (in cases where FXa can be additionally introduced). Therefore, we performed a spiking study to evaluate the FXa removal capacity of our new manufacturing process. The spiking study was conducted using FXa at a concentration of 4.0 μg/mL (concentration in normal plasma) and 21.0 μg/mL (concentration in cryopaste), which were 32.5 times and 169.9 times higher than the concentrations found in normal samples (Fig. 5, Table 1).

The FXa concentration in the specimen spiked with 32.5 and 169.9 times the concentration of FXa as that present in a normal specimen in a production process is shown in Table 1. In this study, when the sample was spiked with FXa at 32.5 times (4.0 μg/mL) the concentration in normal specimens, the amount of FXI/FXIIa remaining after the removal process (post-CEX sample) was found to be below the detection limits (ELISA). This observation confirmed that the manufacturing process could completely remove FXa from the pre-process specimen, even when it is present at a concentration 30 times more than that in a normal

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Fig. 1. FXa remaining after cold ethanol fractionation. The FXa content for each process was calculated using the step residual ratio. 1. Cryo-poor plasma (statistical results of 3 batches, mean ± SD): 100%. 2. Fraction I + II + III paste: 74.4 ± 3.7. 3. Fraction I + III supernatant: 5.8 ± 2.7. 4. Fraction II paste: 5.2 ± 2.1 Results from 3 consecutive batches are shown; all samples were measured at least in duplicate.
Fig. 2. Procoagulant activity, as determined by in-vitro assays performed during cold ethanol fractionation. Since each intermediate product has different IgG concentrations, the results are shown relative to IgG level. Concentration of IgG (mean ± SD): 1. 7.2 ± 0.4 g/L, 2. 5.2 ± 0.2 g/L, 3. 3.8 ± 0.2 g/L, 4. 39.2 ± 2.3. 1. Cryo-poor plasma, 2. Fraction I + II + III paste, 3. Fraction I + III supernatant, and 4. Fraction II paste. 5. Normal citrated plasma for NaPTT. A. ELISA (statistical results of 3 batches, mean ± SD): 1. 478 ± 37.9, 2. 388.2 ± 25.5, 3. 43.1 ± 20.7, 4. 42.5 ± 19.8. B. TGA: 1. 0.4 ± 0.1, 2. 1243.9 ± 219.0, 3. 24.0 ± 15.4, 4. 249.6 ± 124.1. C. Chromogenic FXIa assay: 1. 0.6 ± 0.2, 2. 60.8 ± 17.2, 3. 19 ± 1.1, 4. 4.5 ± 2.2. D. PKA test: 1. 1.69 ± 0.4, 2. 1110.4 ± 235.3, 3. 3.7 ± 2.1, 4. 117 ± 5.5. E. Kallikrein activity measurement: 1. 2.8 ± 0.2, 2. 3.9 ± 0.9, 3. 0.7 ± 0.1, 4. 0.3 ± 0.1. F. NaPTT: 1. 165.1 ± 5.0, 2. 155.8 ± 6.4, 3. 166.4 ± 4.2, 4. 174.9 ± 2.9, 5. 240.1 ± 7.5. Results from 3 consecutive batches are shown; all samples were measured at least in duplicate.

Fig. 3. Removal of coagulation factors (FII, FVII, FIX, FX, and FXIa) by cold ethanol fractionation and CEX chromatography. Loading amount is 5.0 µg of IgG and concentration of FXI/FXIa calculated by ELISA: 1. Cryo-poor plasma (219.0 ng/mL), 2. Fraction I + II + III paste (183.0 ng/mL), 3. Fraction I + III paste (14.0 ng/mL), 4. Fraction II paste (13.0 ng/mL), 5. Pre-CEX chromatography (10.3 ng/mL), 6. Post-CEX chromatography (not detected). A. Image of the western blot with control (Non-reduced condition, 4–20% Tris-glycine gel, upper arrow: IgG monomer, 150 kDa, lower arrow: IgG polymer), B. Band density of FXI/FXIa calculated using Prizm ver. 6.0.
specimen. However, when the specimen was spiked with 21.0 μg/mL of FXIa, a small amount of FXI/FXIa (2.93 ng/mL) remained in the solution. Nevertheless, the residual ratio, based on which the process efficiency is measured, was 0.01%, indicating that a very small percentage of FXIa remained in the sample. Furthermore, FXIa activity, as determined using the TGA, was also found to be below the detection limits (<0.31 ng/mL, as determined by ELISA). This was accomplished by using CEX chromatography with a new resin with high binding capacities for IgG. By adjusting the pH of the elution buffer to 4.5 and conducting ultra/diafiltration, we maximally inhibited IgG polymerization to establish a stable manufacturing process for GC5101B (IgG recovery: 97.7 ± 2.2%, molecular distribution: pre-CEX polymer ratio 0.16 ± 0.04%, post-CEX polymer ratio 0.12 ± 0.05%, purity: 99.5 ± 0.1%) (Table 1). Thus, our method maximized the FXIa removal efficiency during the manufacturing process of GC5101B.

Interestingly, both FXIa and PKA activities substantially increase in Fraction I + II + III as compared to the cryo-poor plasma sample. This is caused due to the activation of FXI to FXIa during the precipitation and filtration step [19–21]. Further, PKA, known as Hageman factor or FXIa, is a type of serine protease that converts Prekallikrein to Kallikrein, which also activates FXI, a Prekallikrein homologue, in the upper level of the intrinsic pathway, or FVII in the extrinsic pathway [22–25]. PKA activation in the plasma is inhibited by anti-thrombin III, C1 inhibitor, α1-antitrypsin, α2-antitrypsin, protease Nexin2, Kunitz-type protease inhibitor [26,27], but during Fraction I + II + III precipitation and filtration, inhibitors such as anti-thrombin III are removed as supernatants, thus allowing PKA activation. The increased levels of PKA activation could be not only due to activated Prekallikrein, but could also be due to activated FXI.

Furthermore, because the amount of FXIa can vary according to the plasma source as a natural result of blood preparation, we also evaluated the robustness of the process through a spiking study. With this spiking assay, we showed that the high binding capacity of resin (>100.0 g/L) further increased the production efficiency. The spiking study including cation exchange chromatography was conducted using 32.5 and 169.9 times the concentration of FXIa as that present in normal specimens, and the results were analyzed using ELISA and TGA. When the sample was spiked with FXIa at 32.5 times the concentration present in normal specimens, the amount of FXIa remaining in the sample after the removal process was below the detection limits. Even when the sample was spiked

### Table 1

<table>
<thead>
<tr>
<th>Process step</th>
<th>Prot. conc. (g/L)</th>
<th>Purity (%)</th>
<th>Polymer contents (%)</th>
<th>Pre-CEX w/o spiked</th>
<th>Pre-CEX w/Spiked</th>
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<td>ELISA (μg/L)</td>
<td>TGA (IU/L)</td>
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<td>0.16</td>
<td>123.6</td>
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<td>0.12</td>
<td>&lt;0.31</td>
<td>&lt;1.56</td>
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All samples were measured at least in duplicate.

a ELISA: Enzyme-linked immunosorbent assay.
b TGA: Thrombin generation assay.
c N.C.: Not calculated.
d These concentrations are 32.5 times and 169.9 times higher than those in normal specimens.
with FXIa at 169.9 times the concentration present in normal specimens, only a small amount of FXI/FXIa could be detected by ELISA; however, by TGA, this amount was found to be below the detection limits (<1.56 mIU/mL). This result was the same regardless of whether the starting material or the spiked samples were tested.

Thus, we successfully removed the coagulation factors FII, FVII, FIX, and FX through cold ethanol precipitation and removed FXIa using chromatography. The use of our method can help ensure the safety of pharmaceutical preparations and by removing FXIa levels to below the detection limit, minimize or even eliminate the risk of thromboembolic events. When consecutive nine batches (on commercial scale) were produced using the new manufacturing process and the procoagulant activity was measured, a very low standard deviation (<5.0%) was found and thus we predict that the risk of thromboembolic events will be low. This confirmed that the
new purification process was robust and that it could ensure the safety of the pharmaceutical preparation.

Several previous studies have attempted to correlate the concentration of coagulant factors in IVIG with the risk of thromboembolic events. For example, Roemisch et al. demonstrated that IVIG samples containing $<2$ mIU/mL did not provoke any thrombus formation using the Wessler test [28]. Funk et al. demonstrated the correlation between NaPTT and thromboembolic events in commercial IVIG batches for a period of 6 years [29]. NaPTT time $>200$ s and a NaPTT ratio $>0.8$ in IVIG resulted in low thromboembolic event rates. In our study, TGA and NaPTT data from GC5101B were found to be below 2 mIU/mL ($<1.56$ mIU/mL) and over 200 s ($262.69 \pm 3.2$). However, our results are difficult to directly compare with the previous studies as we have used different test and standard materials to determine FXa activity. Additional in vivo assays such as the Wessler test would be necessary to demonstrate the safety of the preparations more precisely.

Our findings indicate that the risk of thromboembolic events that can occur with the use of pharmaceutical preparations may be predicted by measuring the procoagulant activities using assays to evaluate thrombin generation, FXa chromogenic activity, Pre-kallikrein activator activity, kallikrein activity, and NaPTT. For this reason, we recommend that the same methods be used to determine procoagulant activity to obtain comparable results. Then, by measuring the correlation between the results of the Wessler test and the procoagulant activity, relatively simple in vitro assay results would be expected to be able to efficiently determine the safety of the product.

Conflict of interest

D. H. Park, G. B. Kang, D. E. Kang, J. W. Hong, and K. Y. Kim are salaried employees of Green Cross Corp. No other potential conflicts of interest are to be declared.

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Author contribution

Dong Hwark Park: Study design and concept, data analysis and interpretation, manuscript writing. Gil Bu Kang: data collection. Dae Eun Kang: data analysis and interpretation. Jeung Woon Hong: data collection. Min Gyu Lee: critical review of the manuscript. Ki Yong Kim: study supervision, critical review of the manuscript. Jeung Whan Han: critical review of the manuscript. All authors have approved the final article.

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References

