Pasteurization Inactivates Clotting Enzymes During Flebogamma and Flebogamma DIF Production

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The authors are employees of Grifols.
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Abstract

Background: A recent thromboembolic adverse events outbreak linked to a specific intravenous immunoglobulin (IVIG) brand has raised serious safety concerns. Methods: Clotting (pro)enzyme activities in the starting fraction (Fr) II+III employed for Flebogamma and Flebogamma DIF (Grifols) production, capacity of purification steps to remove them, capacity of Pasteurisation for (pro)enzymes inactivation and presence of (pro)enzyme activities in final products from different IVIG manufacturers were assessed by well established techniques. Results: Activities in FrII+III ranged from 15-33% (FX) to 76-92% (FXI) and 121-201% (FVII) versus the estimated plasma pool original content. FrII+III showed very short clotting times in NaPTT assay, high PKA and “kallikrein” content, and high thrombin generation capacity. After Pasteurisation, FXII, FXI, FIX, FX, FVII and FII activities in Flebogamma DIF were below the quantitation limit and all tests showed no clotting activity or coagulation activation markers. Two IVIG final products from other manufacturers showed increased thrombin generation capacity and notably shortened clotting times in NaPTT assay (using FXI-deficient plasma). When artificially activated FrII+III samples were spiked into the corresponding production process intermediate, Pasteurisation inactivated all clotting factor activities studied. Conclusion: Pasteurisation step of the Flebogamma and Flebogamma DIF production process inactivates clotting (pro)enzymes and coagulation factor activation markers.

Introduction

Serious safety concerns recently arose worldwide regarding an apparent increased incidence of thromboembolic adverse events (TAEs) associated with the use of an intravenous immunoglobulin (IVIG) brand has raised serious safety concerns. Methods: Clotting (pro)enzyme activities in the starting fraction (Fr) II+III employed for Flebogamma and Flebogamma DIF (Grifols) production, capacity of purification steps to remove them, capacity of Pasteurisation for (pro)enzymes inactivation and presence of (pro)enzyme activities in final products from different IVIG manufacturers were assessed by well established techniques. Results: Activities in FrII+III ranged from 15-33% (FX) to 76-92% (FXI) and 121-201% (FVII) versus the estimated plasma pool original content. FrII+III showed very short clotting times in NaPTT assay, high PKA and “kallikrein” content, and high thrombin generation capacity. After Pasteurisation, FXII, FXI, FIX, FX, FVII and FII activities in Flebogamma DIF were below the quantitation limit and all tests showed no clotting activity or coagulation activation markers. Two IVIG final products from other manufacturers showed increased thrombin generation capacity and notably shortened clotting times in NaPTT assay (using FXI-deficient plasma). When artificially activated FrII+III samples were spiked into the corresponding production process intermediate, Pasteurisation inactivated all clotting factor activities studied. Conclusion: Pasteurisation step of the Flebogamma and Flebogamma DIF production process inactivates clotting (pro)enzymes and coagulation factor activation markers.

Methods

Clotting (pro)enzyme activities in the starting fraction (Fr) II+III used for Flebogamma® and Flebogamma® DIF (Instituto Grifols S.A., Barcelona, Spain) IVIG production, the capacity of purification steps to remove them, the capacity of Pasteurization for (pro)enzyme inactivation, and the presence of (pro)enzyme activities in final products from different manufacturers...
were assessed.  

**Purification processes:** FrII+III was industrially obtained from cryoprecipitate-depleted human plasma (obtained from healthy donors) using Cohn’s method with minor proprietary modifications [9]. The purification processes of Flebogamma® and Flebogamma® DIF have been published elsewhere [10, 11]. Samples of FrII+III industrial extraction as well as samples from intermediate purification stages were analyzed with the methods described below. 

Determination of coagulation (pro)enzymes by one stage clotting assays: Clotting assays [8] were performed in a Q Hemostasis Analyzer (Diagnostic Grifols, Barcelona, Spain) using the corresponding prothrombin time (DG-PT) and APTT (DG-APTT) reagents as well as the factor deficient plasmas from the same manufacturer. FXII, FXI, FIX, FX, FVII and FII were determined in the industrial extraction of FrII+III, as well as in several process intermediates and final products. 

Determination of activated factors (NaPTT): The non-activated partial thromboplastin time (NaPTT) was performed as described in the European Pharmacopoeia (Ph. Eur.) monograph [4] on samples tested either neat or after dilution in the assay buffer. In some experiments the standard method (NaPTT-PPP) was modified using FXI deficient plasma (NaPTT-FXI-Def) in place of the platelet poor plasma (PPP; both types of plasmas from Diagnostic Grifols S.A., Barcelona, Spain). In order to discard the possibility of the inhibitory effects at high IVIG concentrations, the NaPTT was tested on samples either neat or after 1/5 and 1/10 dilutions (FrII+III suspensions were additionally tested at 1/100 dilution) without evidence of inhibitory effects at high IVIG concentrations. For simplicity, illustrations 1 and 3 show only the NaPTT results from neat samples but illustrations 4 and 5 show NaPTT results from samples assayed neat and after 1/5 dilution. 

NaPTT-FXI-Def test was also applied to different lots (n= 1 to n= 4) of six IVIG products from five different manufacturers and concentrations identified as A, B1, B2, C, D and E (all liquid excepting B1, which was lyophilized). The lot from Product A identified as “A 5% (2007)” was manufactured in 2007 and was analyzed one year after its expiration date. The remaining lots were assessed within their shelf life. 

**Presence of thrombin:** Thrombin presence was determined following the Ph. Eur. monograph [6]. Samples were assayed after incubation for 3 h and 6 h at 37°C and for 24 h at 25°C. 

Determination of prekallikrein activator (PKA): Prekallikrein activator (PKA) was determined according to Ph. Eur. monograph [7] using S-2302 (Chromogenix, Milano, Italy) as substrate. 

Determination of Kallikrein and/or “Kallikrein-like substances”: Kallikrein and/or “kallikrein-like” dependent S-2302 hydrolytic activity (“kallikrein”) was determined using the PKA assay. 

Thrombin generation test (TGT): Thrombin generation was determined using the Technothrombin® TGA (Technoclone, Vienna, Austria), with a fluorescence reader and specially adapted software according to the manufacture instructions. Briefly, thrombin generation is initiated in a mixture of platelet poor plasma (PPP) or FXI deficient plasma and sample (32 µl of PPP/FXI deficient plasma and 8 µl of sample) by the addition of 10 µl of a reagent containing a low concentration of phospholipid micelles with 0.5 PM recombinant human tissue factor in Tris Hepes buffer and 50 µl of TGA fluorogenic substrate (0.5 mM Z-G-G-R-AMC, 15 mM CaCl2). For the assay control (vehicle) the test sample is replaced with 8 µl of 0.15 M NaCl. The fluorimetric measurements at 360 nm and 460 nm (excitation/emission) are conducted at 37°C. From the changes in fluorescence over time, the peak concentration of thrombin (nM) in the sample was calculated using a thrombin calibration curve. The value of the vehicle in the same microplate was subtracted from the result of each test sample. 

TGT-FXI-Def test was also applied to different lots (n= 1 to n= 4) of products of manufacturers A, B, C, D and E. 

Laboratory preparation of FrII+III spiking suspensions: Laboratory FrII+III suspensions were used as spikes for the industrial materials before acid pH treatment or Pasteurization, in order to investigate the capacity of these steps in the inactivation of clotting (pro)enzymes. 

Samples of FrII+III were suspended in a ratio 0.3g FrII+III/ml of water and diluted 1: 4 in 0.15 M NaCl. After pH adjustment (7.3-7.4 with 0.1 N NaOH), the suspension was incubated for 60 min. at 30°C under gentle stirring, further diluted 1: 1 in 0.15 M NaCl, incubated again for 90 min (under gentle stirring) at 30°C and filtered through a 0.45 µm pore filter. These activated FrII+III derived suspensions were spiked (v/v) 1/5, 1/10 and 1/20 in samples taken from the manufacturing intermediates (containing about 99% pure IgG) before acid pH (4.5 hours, pH 4.0, 37°C) treatment or before Pasteurization (10h, 60°C) were applied to the corresponding mixtures in the laboratory. 

**Results**

Coagulation (pro)enzyme content and activation status of industrially re-suspended FrII+III: Assuming 100% from the expected content of all coagulation enzymes...
proenzymes in the normal human plasma pool used for the preparation of Frll-III, the total recovery of activities in the industrially re-suspended Frll-III (one-stage clotting assays on frozen samples with an average OD280 of 56.6±10.4, n=2) ranged from 15-33% for FX to 121-201% for FVII, with intermediate values for the remaining activities tested: 35-40% for FXII, 61-68% for FII, 67-70% for FIX and 76-92% for FXI.

Re-suspended Frll-III showed very short clotting times in the NaPTT-PPP assay, high PKA and “kallikrein” content, as well as high thrombin generation capacity (Illustration 1).

Follow-up of coagulation activities during the purification process: With the only exception of FXI (Illustration 1), all remaining clotting factor activities studied were below the quantitation limit of the corresponding one-stage assays in all of the Flebogamma® DIF process intermediates studied, including the first gross purification step after industrial extraction (PEG precipitation).

FXI clotting activity in Frll-III dropped from 71-88 IU/g of total protein, to 0.4-1.1 IU FXI/g protein after PEG precipitation, DEAE chromatography purification and concentration by ultrafiltration. After acid pH treatment, FXI was still detectable in two of the three processes analyzed. However, after Pasteurization FXI activity was always below the quantitation limit, even in the most concentrated samples (10 % final bulk product before dosage in vials, see Illustration 1). “Kallikrein” activities and thrombin generation capacity could be observed in some samples even after DEAE chromatography purification, including the acid pH treated intermediate. However, after Pasteurization all tests showed negative results for clotting activity and for coagulation activation indicators (Illustration 1).

Final product results: One to four lots of Flebogamma® 5% DIF were analyzed by one-stage clotting assays and using clotting factor activation tests (thrombin presence, NaPTT-PPP, NaPTT-FXI-Def, PKA, TGT-PPP or TGT-FXI-Def) with negative results. In addition, Flebogamma® 10% DIF and Flebogamma® 5% were tested by TGT-FXI-Def (Illustration 2) while Flebogamma® 5% was also tested by NaPTT-FXI-Def (Illustration 3) with no indication of clotting factor activation in any test. Also in Illustration 2, products A and B1 from two other manufacturers showed markedly increased thrombin generation capacity (TGT-FXI-Def) compared to the vehicle or to the rest of products. As seen in Illustration 3, the same products (A and B1) showed notably shorter clotting times in the NaPTT-FXI-Def assay, in contrast to the majority of products, that showed results comparable to the assay control.

Capacity of Pasteurization and acid pH treatment to inactivate procoagulant activities: Samples of industrial materials before acid pH treatment or before Pasteurization spiked with activated Frll-III (see methods) showed positive results when assayed for coagulation activation markers. The acid pH treatment showed a very limited effect on reducing the markers even in the lowest spike proportion, with the exception of PKA (Illustration 4), whereas Pasteurization rendered the mixtures negative for all tests performed, even with the highest spike volume (Illustration 5).

Discussion

The apparent TAEs outbreak linked to a specific IVIG product [1], has created a great deal of anxiety to patients receiving IVIGs, their caregivers, health authorities and IVIG manufacturers. However, this situation seems to be an isolated, brand-dependent outbreak, apparently due to a modification in the production process [1]. An unforeseen consequence appears to be the increase in FXIa (and possibly other impurities) in the product [1].

The presence of coagulation factors in IVIG was previously described in several reports [3] in which FXIa was postulated to play a significant role in arterial and venous thrombosis [12] and in TAEs potentially associated with IVIG [3], although other impurities cannot be excluded [1]. Our results agree with the previously known presence of coagulation factors in Frll-III (or Frll-II+III, as used by some manufacturers), the starting fraction for IVIG production [9, 13]. The amount of FVII activity detected in Frll-III by clotting assay (about 200% estimated recovery from the plasma pool in one of the processes) indicates a relevant degree of FVII activation. The detection in Frll-III of shortened NaPTTs, high PKA values, “kallikrein” activities and positive TGT results strongly suggest some degree of activation for all coagulant proenzymes.

Manufacturing IVIGs from Frll-III (or Frll-II+III) requires a precipitation step for initial gross purification. Ethanol, Caprylate or PEG are usually employed as precipitating agents [14-16]. The initial purification is then followed by a polishing step, generally performed with IEC [14]. The combination of these steps must yield at least 95% IgG purity in order to comply with the Ph. Eur. monograph [17]. IEC is generally performed with an anion exchanger at slightly acidic pH (e.g., between 5.2 and 6.0), to allow IgG (charged positively, pi: 6.3–7.3) to flow through the resins while main residual impurities (charged mostly negatively, pi:
2.7–5.5) [18] remain bound to the resin. However, the anion exchange chromatography has a limited (or null) capacity to remove positively charged impurities such as FXI (or FXIa, both with pIs near 9.1) by direct adsorption [19, 20]. According to our results, another positively charged protein in FrII+III (or FrI+II+III) is thrombin (pI: 7.0–7.6) [21]. An increase in the amount of FXIa, thrombin or other procoagulant substances in the starting fraction for IVIG production (e.g., because of a switch from FrII+III to FrI+II+III) may overcome the purification capacity of the combined precipitation plus IEC steps (if the latter is applied), increasing the thrombogenic potential of the IVIG. Fraction I is rich in the proteins involved in coagulation [15]. Another step commonly applied to IVIGs is acid pH treatment. Our experiments show a very limited capacity of short term acid pH treatment to inactivate clotting enzymes.

Although less commonly employed in the production processes of IVIGs, Pasteurization has an important heat-denaturation capacity for proteins and viruses. In fact, among the main IVIGs currently marketed in Europe and/or USA, only Flebogamma® and Flebogamma® DIF include the Pasteurization step. Our data demonstrates that, even in well adjusted purification processes starting from FrII+III (where IgG is purer than in FrI+II+III), traces of FXI (or FXIa) may be detected after the initial precipitation and IEC steps, and also resist short term acid pH treatment. However, no residual FXIa can be measured after Pasteurization, even when the product is concentrated to 10%.

Activated FrII+III was spiked (up to a 20% v/v) in our laboratory into the industrial process material before Pasteurization. No residual procoagulant activity could be detected in any of the wide array of clotting tests applied after submitting these samples to Pasteurization in the laboratory. Therefore, Pasteurization has relevant capacity for (activated or not) clotting enzyme inactivation, well above the real levels of impurities present in Grifols’ IVIG processes before this production step. When these same clotting tests were applied to final products from different manufacturers, no indication of the presence of coagulant factors or of coagulant factor activation markers was observed for any product tested, with two exceptions. The FXI deficient plasma-dependent TGT and NaPTT results appear to distinguish between Flebogamma® and Flebogamma® DIF and several other products (with results at the same level of the assay vehicle) on one side and products A (even 3 years after manufacture in one of the studied lots) and B1, which showed clear indications of the presence of clotting factors, most probably FXI and FXIa, although other relevant impurities cannot be ruled out with these assays.

These results concerning the efficacy of Pasteurization in Flebogamma® and in both 5% and 10% Flebogamma® DIF are concordant with the Pharmacovigilance data. Approximately 49 million g of Flebogamma® have been marketed worldwide since it was first introduced in February 1993 up to September 2010. Only eight infusions suspected to be related to TAEs have been reported up until 12th October 2010 (data on file, Instituto Grifols S.A.). Therefore, the overall report rate can be estimated as 0.16 (1 case in 6.1 million g). As for Flebogamma® 5% DIF, since May 2007 (initial marketing date) up until September 2010 approximately 12.0 million g were marketed. Only two infusions suspected to be related to TAEs have been reported up until 12th October 2010 (data on file, Instituto Grifols S.A.). The overall report rate can be estimated at 0.17 (1 case in 6.0 million g), coinciding with the Flebogamma® data, in spite of the very different span of the marketing period.

In contrast, according to published information [1], the report rate of TAEs with the brand involved in the apparent TAEs outbreak was around 0.67 between 2005 and 2007 (1 case per 1.5 million g) while in 2008 and 2009 the rate increased to around 2 (1 per 0.5 million g). Remarkably, from January to July 2010, the report rate of TAEs increased to around 5.5 (1 per 0.18 million g).

In summary, our data show that the starting fractions used for IVIG production contain relevant amounts of coagulation proenzymes and strongly suggest they may be activated to some extent. The common purification steps (initial precipitation of impurities followed by IEC) as well as short term acid pH treatment have limited capacity to separate or inactivate some of these impurities. However, Pasteurization consistently inactivated all marker of coagulation enzymes presence. Finally, our data appear to distinguish two groups of products, one with high thrombin generation capacity and with short NaPTT times (products A and B1), and the other with results comparable to the corresponding assay controls (products B2, C, D, E, Flebogamma® and Flebogamma® DIF).

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Illustrations

Illustration 1

Flebogamma® DIF purification process follow-up

<table>
<thead>
<tr>
<th></th>
<th>Resuspended FrII+III (n=4)*</th>
<th>PEG 4% filtrate (n=3)*</th>
<th>DEAE effluent (n=3)*</th>
<th>Concentrated ultrafiltrate I (n=3)*</th>
<th>After acid pH treatment (n=3)*</th>
<th>After Pasteurization (n=3)*</th>
<th>Concentrated final bulk (n=3)§*</th>
</tr>
</thead>
<tbody>
<tr>
<td>FXI: C (IU/g protein)</td>
<td>71-88</td>
<td>NQ</td>
<td>NQ</td>
<td>0.4-1.1</td>
<td>NQ-0.8</td>
<td>NQ</td>
<td>NQ</td>
</tr>
<tr>
<td>NaPTT-PPP‡ (s)</td>
<td>17-135</td>
<td>219-241</td>
<td>218-288</td>
<td>197-213</td>
<td>220-250</td>
<td>225-336</td>
<td>225-299</td>
</tr>
<tr>
<td>PKA (IU/ml)</td>
<td>137-3977</td>
<td>34-143</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
<td>&lt;2</td>
</tr>
<tr>
<td>&quot;Kallikrein&quot;</td>
<td></td>
<td></td>
<td>0.001-0.00</td>
<td>0.003-0.00</td>
<td>0.017-0.030</td>
<td>0.013-0.02</td>
<td></td>
</tr>
<tr>
<td>(ΔAU/min)</td>
<td>0.14-1.40</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>TGT (nM)*</td>
<td></td>
<td></td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>PPP</td>
<td>199</td>
<td>12</td>
<td>-8</td>
<td>56</td>
<td>19</td>
<td>-41</td>
<td>-56</td>
</tr>
<tr>
<td>FXI-Def</td>
<td>474±57</td>
<td>53±15</td>
<td>41±14</td>
<td>141±14</td>
<td>135±39</td>
<td>2±3</td>
<td>3±10</td>
</tr>
</tbody>
</table>

*TGT-PPP (n=1); TGT-FXI-Def (n=3). Negative results indicate results below the value obtained for the vehicle.
§5% bulk (n=1) plus 10% bulk (n=2).
‡NaPTT results from undiluted samples (the assay control buffer) for these assays ranged 237-301 seconds. When n>1, results are expressed as range or as mean ± SD; NQ: Non-quantifiable.
Illustration 2

Thrombin generation test (TGT; mean ± SD) assays with FXI deficient plasma, in products from different manufacturers (A, B1, B2, C, D, E and F). The lot identified as "A 5% (2007)" was manufactured in 2007.
Illustration 3

Non-activated partial thromboplastin time (NaPTT; mean ± SD) assays with FXI deficient plasma in products from different manufacturers (A, B1, B2, C, D, E and F). The lot identified as "A 5% (2007)" was manufactured in 2007.
Illustration 4

Effect of acid pH treatment on activated clotting factors

<table>
<thead>
<tr>
<th>Acid pH treatment (n=1)</th>
<th>Spike 1/20</th>
<th>Spike 1/10</th>
<th>Spike 1/5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>NaPTT-PPP (s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neat</td>
<td>54</td>
<td>136</td>
<td>34</td>
</tr>
<tr>
<td>Diluted 1/5</td>
<td>119</td>
<td>225</td>
<td>82</td>
</tr>
<tr>
<td>Control (buffer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKA (IU/ml)</td>
<td>5</td>
<td>&lt;2</td>
<td>24</td>
</tr>
<tr>
<td>&quot;Kallikrein&quot; (ΔAU/min)</td>
<td>0.019</td>
<td>0.019</td>
<td>0.020</td>
</tr>
<tr>
<td>TGT FXI-Def Plasma (nM)</td>
<td>286</td>
<td>239</td>
<td>323</td>
</tr>
</tbody>
</table>
Illustration 5

Effect of Pasteurization on activated clotting factors

<table>
<thead>
<tr>
<th>Pasteurization (n=3)</th>
<th>Spike 1/20</th>
<th>Spike 1/10</th>
<th>Spike 1/5</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
<td>Before</td>
</tr>
<tr>
<td>NaPTT-PPP (s)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neat</td>
<td>53-133</td>
<td>269-291</td>
<td>36-99</td>
</tr>
<tr>
<td>Diluted 1/5</td>
<td>97-224</td>
<td>236-293</td>
<td>62-190</td>
</tr>
<tr>
<td>Control (buffer)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PKA (IU/ml)</td>
<td>4-28</td>
<td>&lt;2</td>
<td>18-65</td>
</tr>
<tr>
<td>&quot;Kallikrein&quot; (ΔAU/min)</td>
<td>0.010-0.011</td>
<td>0.001-0.002</td>
<td>0.012-0.014</td>
</tr>
<tr>
<td>TGT (nM)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPP</td>
<td>81</td>
<td>-33</td>
<td>70</td>
</tr>
<tr>
<td>FXI-Def Plasma</td>
<td>387±72</td>
<td>40±37</td>
<td>419±64</td>
</tr>
</tbody>
</table>

Results are expressed as range or average ± SD. Negative results indicate results below the value obtained for the vehicle.

TGT-PPP data from n=1
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