A MANUFACTURING PROCESS TO OBTAIN A HIGHLY-PURIFIED TRIPLE-SECURED FIBRINOGEN PRODUCT

R&D Area, Instituto Grifols S.A.
Parets del Vallès, Barcelona, Spain
Fibrinogen Concentrate

Introduction

Human Fibrinogen is the principal protein of vertebrate blood clotting.
Acquired or congenital deficiencies (afibrinogenemia) can lead to bleeding complications.
Fibrinogen Concentrate

Introduction

- Fibrinogen is a high molecular weight (340 KDa) glycoprotein synthesised in the liver with a concentration in blood plasma is around 3 g/L.

- Fibrinogen, is a hexamer containing two sets of three different chains (α, β, and γ), linked to each other by disulfide bonds.

\[ \text{~45nm} \]
Instituto Grifols aimed to develop a high purity Human Fibrinogen concentrate incorporating state-of-the-art safety procedures while maintaining its natural properties.

- **PLASMA**
  - **Fr-I**
    - **Suspension/Clarification**
    - **SD TREATMENT** (6h at 27ºC)
    - **Glycine precipitations**
    - **Suspension/Clarification**
  - **FreezeThaw/Clarif./Dilution**
  - **NANOFILTRATION** (35 nm & 20nm)
  - **Ultra/diafiltration** (formulation)
  - **Sterile Filling/Freeze-dry**
  - **HEAT TREATMENT** (2h at 101ºC)
Fibrinogen Concentrate

Purification steps

• Fibrinogen is purified from Cohn’s Fraction I suspension by three sequential precipitation steps

• 3er precipitate is reconstituted and clarified

Cohn’s Fraction I

SOLVENT-DETERGENT TREATMENT

1st Glycine precipitate

2nd Glycine precipitate

3rd Glycine precipitate

3rd Glycine precipitate reconstituted and clarified (Frozen solution)
Fibrinogen Concentrate
Precipitation steps

Cohn's Fraction I

SOLVENT-DETERGENT TREATMENT

1st Glycine precipitate
2nd Glycine precipitate
3rd Glycine precipitate
3rd Glycine precipitate reconstituted and clarified (Frozen solution)

Sodium citrate-chloride extraction

1% Polysorbate 0.3% TnBP

Glycine 1.7M
Glycine 1.5M
Glycine 1.5M
Clarification Freeze-Thawing Filtration
## Fibrinogen Concentrate

### Precipitation steps

<table>
<thead>
<tr>
<th></th>
<th>Mass ratios (%)</th>
<th>Recovery (%)</th>
<th>Yield (g/kg Fr-I)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Clottable Fibrinogen</td>
<td>Fibrinogen Antigen</td>
</tr>
<tr>
<td>Cohn’s Fraction I Extraction</td>
<td>--</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>1st Glycine precipitate</td>
<td>100</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>2nd Glycine precipitate</td>
<td>99.7</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3rd Glycine precipitate</td>
<td>96.9</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>3rd Glycine precipitate</td>
<td>--</td>
<td>78.2</td>
<td>64.9</td>
</tr>
</tbody>
</table>

3rd Glycine precipitate reconstituted and clarified
## Fibrinogen Concentrate

### Precipitation steps

<table>
<thead>
<tr>
<th></th>
<th>Ratio Clottable/Antigen Fibrinogen (%)</th>
<th>Purity (electrophoresis) (%)</th>
<th>Ratio Fibronectin/ Fibrinogen (mg/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cohn’s Fraction I Extraction</td>
<td>99.7</td>
<td>76.4</td>
<td>51</td>
</tr>
<tr>
<td>3rd Glycine precipitate reconstituted and clarified</td>
<td>100</td>
<td>100.0</td>
<td>10</td>
</tr>
</tbody>
</table>
Final steps

- Diluted solution is nanofiltered by double nanofiltration 35nm & 20nm
- Finally product is concentrated and formulated

3rd Glycine precipitate reconstituted and clarified (Frozen solution)

Diluted solution

35nm & 20nm NANOFILTRATION

Concentrated and Formulated Solution

Sterile Filtration / Filling

Frezze drying / Heat Treatment
Fibrinogen Concentrate

Final steps

3rd Glycine precipitate reconstituted and clarified (Frozen solution)

Diluted solution

35nm + 20nm NANOFILTRATION

Concentrated and Formulated Solution

Sterile Filtration / Filling
Frezzing/Thawing

FIB < 1.5mg/ml

35nm & 20nm NANOFILTRATION

4%

Fibrinogen

Frezze drying / Heat Treatment 101°C / 2 hours
### Nanofiltration Development

- **Effect of intermediate freezing/thawing**

<table>
<thead>
<tr>
<th>Process</th>
<th>Thawing temperature (°C)</th>
<th>Weight of insoluble material (kg)</th>
<th>OD (280) prior to freezing (AU)</th>
<th>OD (280) after freezing and filtration (AU)</th>
<th>Difference in OD (AU)</th>
<th>% Protein recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 - 10</td>
<td>2.0</td>
<td>ND</td>
<td>ND</td>
<td>NA</td>
<td>NA</td>
</tr>
<tr>
<td>2</td>
<td>30.5</td>
<td>0.1</td>
<td>47.8</td>
<td>46.5</td>
<td>1.3</td>
<td>97.5</td>
</tr>
<tr>
<td>3</td>
<td>9 ± 1</td>
<td>1.0</td>
<td>45.2</td>
<td>37.5</td>
<td>7.7</td>
<td>83.0</td>
</tr>
<tr>
<td>4</td>
<td>19 ± 1</td>
<td>0.5</td>
<td>39.5</td>
<td>34.3</td>
<td>5.2</td>
<td>86.8</td>
</tr>
<tr>
<td>5</td>
<td>7 ± 1</td>
<td>1.9</td>
<td>35.0</td>
<td>24.5</td>
<td>10.5</td>
<td>70.0</td>
</tr>
<tr>
<td>6</td>
<td>11 ± 2</td>
<td>0.9</td>
<td>36.0</td>
<td>31.5</td>
<td>4.5</td>
<td>87.5</td>
</tr>
</tbody>
</table>
Nanofiltration Development

- Effect of intermediate freezing/thawing

<table>
<thead>
<tr>
<th>Process</th>
<th>Thawing temperature (°C)</th>
<th>Production capacity (kg solution/m²)</th>
<th>Filtration time (h)</th>
<th>Filtration flow rate (kg/m²/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>5 - 10</td>
<td>&gt; 43.8</td>
<td>3.0</td>
<td>14.6</td>
</tr>
<tr>
<td>3</td>
<td>9 ± 1</td>
<td>&gt; 56.3</td>
<td>6.5</td>
<td>8.7</td>
</tr>
<tr>
<td>4</td>
<td>19 ± 1</td>
<td>&gt; 51.7</td>
<td>8.5</td>
<td>6.1</td>
</tr>
<tr>
<td>5</td>
<td>7 ± 1</td>
<td>&gt; 27.1</td>
<td>3.0</td>
<td>9.0</td>
</tr>
<tr>
<td>6</td>
<td>11 ± 2</td>
<td>&gt; 32.8</td>
<td>3.8</td>
<td>8.6</td>
</tr>
</tbody>
</table>
Nanofiltration

- Development 20nm nanofiltration*
  - Freezing
  - Thawing at defined temperature (5-20ºC)
  - Separating undisolved materials
  - Diluting to optimal concentration (FIB < 1.5mg/ml)

- Conditions:
  - Arginine 2% pH 7.0 / 30ºC

- Final tandem:
  - Asahi P35N + P20N

* US7442308 Patent
Manufacturing Scale

Flow decay Consistency (n=5)

Amount Filtered Solution (L/m²)

Flow decay (%)
Manufacturing Scale

Productivity Consistency (n=5)

Amount Protein Filtered (g/m²)

Time (min)


Nanofiltration

- **Virus Safety Validation**
  - Reduction factors (log10/ml)

<table>
<thead>
<tr>
<th>Target</th>
<th>HBV/Herpes</th>
<th>HIV 1 and 2</th>
<th>HCV/WNV</th>
<th>HAV</th>
<th>PPV</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Model</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PRV (env. DNA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>180 - 200 nm</td>
<td>≥ 6.09</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HIV 1 (env. RNA)</td>
<td></td>
<td>≥ 5.57</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BVDV (env. RNA)</td>
<td></td>
<td></td>
<td>≥ 4.53</td>
<td></td>
<td></td>
</tr>
<tr>
<td>40 - 60 nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HAV (non-env. RNA)</td>
<td></td>
<td></td>
<td></td>
<td>5.22</td>
<td></td>
</tr>
<tr>
<td>27 - 32 nm</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B19 Virus</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>4.37</td>
</tr>
<tr>
<td>(non-env. DNA)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>18 - 26 nm</td>
<td></td>
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</tr>
</tbody>
</table>

Nanofiltration (Planova® filters, 35 nm & 20 nm)
Specific pathogen safety steps

- Treatment with **Solvent Detergent**
  (0.3 % TNBP + 1 % Polisorbate 80, 6-6.5 hours).

- **Nanofiltration**: Planova 35N & Planova 20N.

- Freeze-Dry and **Heat treatment** (101 ± 1 ºC, 2 - 2.5 hours).
Final Product
Intravenous Fibrinogen

• Main Features:
  - Freeze-dried 1g dose size presentation
  - Filled 4% in vials
  - Soluble (2%) in ~10 min at 20-25°C
Final Product 2%

<table>
<thead>
<tr>
<th></th>
<th>Final Vial Heat Treated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Clottable Fibrinogen</strong></td>
<td></td>
</tr>
<tr>
<td>Concentration (mg/ml)</td>
<td>19 ± 1</td>
</tr>
<tr>
<td>Specific Activity (mg/mg protein)</td>
<td>0.56 ± 0.02</td>
</tr>
<tr>
<td>Ratio Clottable/Antigen (%)</td>
<td>84.9 ± 3.6</td>
</tr>
<tr>
<td><strong>Fibrinogen Antigen</strong></td>
<td></td>
</tr>
<tr>
<td>Concentration (mg/ml)</td>
<td>22 ± 1</td>
</tr>
<tr>
<td><strong>Purity</strong></td>
<td></td>
</tr>
<tr>
<td>(% electrophoresis)</td>
<td>64.01 ± 1.45</td>
</tr>
<tr>
<td><strong>Fibronectin</strong></td>
<td></td>
</tr>
<tr>
<td>(mg/g Fibrinogen)</td>
<td>4.0 ± 1.0</td>
</tr>
</tbody>
</table>
Final Product

Conclusion

• The optimized process developed for fibrinogen nanofiltration performs very consistently in manufacturing scale.

• The designed manufacturing process allows to obtain a highly-purified triple-secured Fibrinogen product.
Final Product

Conclusion

• Nanofiltration developments should evaluate aspects like:
  - Conductivity, pH
  - Viscosity, temperature
  - Optimal concentration
  - Protein stability
  - Specific behavior of your protein