A report from the first Plasma Products Biotechnology meeting

Daydream Island
Queensland
Australia
March 27-30, 1999
From the chairman.

Plasma products - where are we and where are we going?

Production of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris*.

Chromatographic albumin production - pilot to manufacturing plant.

Negatively-charged albumins: a novel class of polyanionic proteins with potent anti-HIV activity.

Development and production of coagulation factor concentrates: threats or opportunities?

Evolution of coagulation factor concentrates: biotechnological breakthrough of immunoaffinity chromatography using monoclonal antibodies.

Immunogenicity of heat-treated therapeutic plasma derived products.

Plasma fractionation based on chromatography and precipitation by polyethylene glycol and caprylic acid.

Development and scale up of a production-scale chromatographic process for the production of human IgG.

Chromatographic purification of immunoglobulins.

Characterization and formulation development of a new liquid intravenous immunoglobulin: Vigam Liquid.

Chromatographic partitioning of model and relevant blood borne viruses and of immune complexes of relevant viruses in the manufacture of albumin.

Cofact, a double virus inactivated prothrombin complex concentrate.

The INACTINE technology - an advance in the inactivation of non-enveloped and enveloped viruses.

Current perspectives on recombinant plasma products - a regulator’s view.

New plasma products - opportunities and challenges.

The front cover shows a number of participants gathered on the beach on Daydream Island, Australia.

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This special issue of Downstream contains synopses of a number of papers given at the first Plasma Products Biotechnology (PPB) meeting, held in Australia in March 1999. This meeting brought together some of the most experienced people working and researching within the plasma industry. The purpose of the meeting was to offer a forum for the open exchange of information, data and ideas, and to give participants, coming from all corners of the industry, from research to manufacturing to regulatory, the opportunity for peer interaction and networking.

The meeting was co-sponsored by Amersham Biosciences, Sweden and CSL Ltd, Australia. CSL develops, manufactures and markets pharmaceutical products of biological origin. Its Bioplasma division is the exclusive manufacturer in Australia of products derived from human plasma, supplying the domestic Australian market as well as many other countries. At its Broadmeadows site on the outskirts of Melbourne, it has built a specially designed facility for plasma fractionation which incorporates some of the latest technologies for plasma products. Here, CSL has introduced a chromatographic process for the manufacture of albumin which gives a product with a purity higher than that achievable with the standard Cohn fractionation process.

At the four-day meeting, presenters spoke about the different issues and developments affecting the industry. It was the emphasis on chromatographic processing that lead to the idea to produce a special edition of Downstream based around this theme. The articles you will read here have been selected by us for what we believe to be of interest to readers of Downstream magazine. They are just a few of the approximately fifty, highly informative and well-delivered presentations, many of which lead to some very lively discussions and gave food for thought for the future development of the plasma industry.

My thanks go to all presenters, participants, fellow organizers and our co-sponsor, CSL, for making this a truly successful meeting. I look forward to seeing you all again in 2001.

Jan Berglöf
Chairman of the Organizing Committee
Plasma products - where are we and where are we going?

Summary of the plenary lecture given by Dr Richard Walker, BioProducts Laboratory, Elstree, UK

Five to six years ago there were lots of commercial and not-for-profit manufacturers in mainly North America and Europe, and there were many very small and poor quality manufacturers in other parts of the world. Regulations were limited both in Europe and the United States. Few blood centres in Europe were properly licensed by the local regulators. The situation in the USA was no better. The past 5 years has witnessed substantial consolidation of manufacturers in both the commercial and the not-for-profit sectors. The driving forces in the commercial sector have been the squeeze on margins, and cost increases due largely to regulatory pressures. Overall capacity across the world has increased by 4 million litres/year. The not-for-profit sector has undergone the greatest change. New processes have been introduced, and many fractionators have moved from a position of having no product licences and no clinical data, to where all products are properly licensed.

Product safety has probably been the biggest driver for the whole industry with four particular issues predominant, namely, plasma quality, virus elimination, clinical trial requirements and GMP enforcement.

• There are still concerns about the unknown viruses, but with the world today more sensitive to potential contaminants of blood one hopes that new problems can be identified earlier.

• Virus elimination has been a major challenge. The major pathogenic viruses have generally been dealt with. European regulator demands for a second virus elimination step for non-enveloped viruses, however, have not yet been fully met.

• Clinical trial developments have been a source of debate. In the USA, the FDA does not require clinical trials before introducing a virus filtration step; in Europe clinical trials are required, which can cause delay.

• In Europe, discussion between the industry and regulators has developed from no dialogue prior to implementation of regulations to close and effective working between the parties. In the USA the situation has developed differently with a very much stronger consumer input.

On the product front, a recombinant Factor VIII and a recombinant Factor IX have been introduced. Factor VIII, which was the driving force for plasma demand, is being replaced in this role by intravenous immunoglobulin.

The biggest threat is perhaps new variant CJD. In the UK, the precautions taken have meant nofractionation of any UK plasma. Will a diagnostic test be developed before nvCJD appears elsewhere and will the test be sufficiently sensitive to levels of prion below that at which infection is transmissible, if it can be found in blood at all? A lot of research is going on to demonstrate that fractionation processes can remove prion protein if it does get into the blood supply.

The Future

Will we still have a thriving plasma business in 5 years time? What changes are we likely to see? The clinical efficacy of plasma products will be under continuing and increasing focus and there will certainly be more litigation. The problems generated by Hepatitis C and HIV in blood products continue to rage across the world and patient groups are requesting better information about the products they use at home. There will also be more pressure on virus elimination.

Recombinant Factor VIII and Factor IX are increasing in major developed markets, despite the fact that they are expensive and, in the case of recombinant Factor IX, usage levels have to be much higher to achieve an equivalent dose. Albumin is also under pressure. Some manufacturers already have additional products to their mainstream, such as ATIII and alpha 1 antitrypsin, but demonstrating clinical efficacy in anything other than protein deficiency is difficult.

Other recombinant products being developed include albumin, fibrinogen and Factor XIII. But recombinants are under threat from transgenics (or vice versa) where similar products are in development. For instance, there are two transgenic fibrinogens on the way. Costs are an issue with both technologies. Transgenics also pose questions for the regulators on how to deal with potential animal viral risks and what clinical trials will be required to demonstrate safety and efficacy.

Then there is gene therapy. While gene therapy is obviously the ultimate solution for protein deficiency, the time-scale is likely to be very long.

All these developments will generate a particular problem for plasma product manufacturers with regard to clinical trials for new products or licence variations. There is a limited number of patients on which to test products and many clinicians (and patients) will be attracted to non-plasma derived alternatives.

Conclusions

In the next five years I believe that world wide demand for plasma derivatives will have grown steadily, with the US and Europe still being the dominant users. Intravenous IgG will be the driving force and there will be further consolidation of manufacturers. New virus elimination processes will be established, particularly if they are effective for non-enveloped as well as enveloped viruses.

At least one transgenic product will be licensed with others under clinical trials. Gene therapy for Factor IX deficiency will be close. Plasma products will continue to be sold in parallel in the same markets as alternatives, provided there are no major scares and nvCJD does not spread outside the UK.

On the regulatory side, more harmonization with fewer new regulations, and more uniform policing of existing ones.
Production of recombinant human serum albumin from the methylotrophic yeast *Pichia pastoris*

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To date, human serum albumin (HSA) has been produced by the fractionation of whole blood. Because the source of the blood can vary, there is always the potential risk of contamination of the HSA product by blood-derived pathogens. Human plasma is in limited supply in Japan and only about 26% of the Japanese produced albumin is obtained from domestic plasma. The development of an alternative method for the industrial preparation of HSA is therefore desired since it would greatly assist in the general movement towards Japan’s self-sufficiency in blood and blood products.

Recombinant DNA technology is expected to play an important role in such situations. However, the large-scale production of a pharmaceutical grade of recombinant HSA (rHSA) presents a number of problems for recombinant protein technology. rHSA is unlike the other blood proteins and other recombinant proteins in that the molecule is structurally complex, it has a low unit price, and it has a large market volume. Furthermore, HSA is used clinically to treat severe hypoalbuminemia or traumatic shock and the usual dosages of HSA are in excess of 10 g/dose. Thus, the process for developing a rHSA must produce an extremely pure product, in especially large-scale production.

We have used the methylotrophic yeast *Pichia pastoris* as a host strain for the rHSA expression and then developed the fermentation and purification processes. Our fermentation process uses methanol feeding for the secretion of rHSA into the culture broth. By optimizing the physiological and environmental aspects of the fermentation, the production level of the rHSA exceeded 10 g-rHSA/l.

After fermentation, cell separation processes such as centrifugation and filter press have been used in the common purification process. However, there are some disadvantages with these mechanical separation procedures. For example, the bowl needs to be changed frequently and/or microorganisms are exposed to the working environment. Furthermore, mechanical stress may cause destruction of the cells, which results in increased levels of cell-derived contaminants. To overcome these problems, our purification process employs STREAMLINE™ expanded bed adsorption for direct capture of the target product. Culture fluid containing yeast cells was applied directly to the STREAMLINE expanded bed column. rHSA was adsorbed to the column, while yeast cells and impurities passed through, collected, sterilized and finally discarded (Figure 1). Direct connection of STREAMLINE with the fermentor allows the construction of a closed system to isolate the recombinant microorganisms in the restricted apparatus.

HSA does not possess any distinct biological activity like enzymes or cytokines. Following purification by a combination of several chromatographic and membrane filtration techniques, the purified rHSA was thus analyzed for its structure and purity. The similarity of rHSA to plasma-derived HSA was determined using various structural analyses. From X-ray crystallographic analysis three-dimensional models of rHSA and plasma-derived HSA were obtained which showed conclusively that the structure of albumin types was identical.

To verify the removal of yeast-derived impurities in purified rHSA, a highly sensitive immunoassay (EIA) for the detection of low levels of impurities in the presence of high concentrations of recombinant product was established. The detection limit of this EIA method was 1 ng of yeast derived impurities/ml. Contamination with yeast-derived impurities could not be detected in the purified rHSA at a concentration of 250 mg rHSA/ml. Thus, rHSA was intensively assessed for its identicalness to the plasma-derived HSA and for the absence of impurities in pre-clinical tests. In the clinical studies, emphasis was placed on the safety aspects of the administered rHSA.

After confirmation of the safety and efficacy in the clinical trials, we submitted an application to the Japanese Ministry of Health and Welfare in 1997 to manufacture rHSA in Japan. Large-scale facilities for the commercial production of rHSA have been constructed in Hokkaido, Japan. These facilities are now being validated.
The CSL Ltd Bioplasma Division Plant is located at Broadmeadows (Figure 1) just outside Melbourne, Australia. The plant has been fully operational since June 1995 and contains the largest chromatographic albumin plant in the world.

In the early 1980s it was obvious that many new plasma products were being based on chromatographic processes, utilizing either plasma or Cohn intermediate as the starting material. Chromatographic process technology had reached the stage where albumin fractionation could be considered on a manufacturing scale. Consequently a development project was initiated with the view to developing a robust chromatographic albumin fractionation process. Shortly thereafter, a decision was made by CSL to build a new plasma fractionation facility. This would ultimately lead to the adoption of the chromatographic albumin process currently in use today. Over a period of 10 years, multiple trials were performed to establish and refine the process, prepare stability trials, and ultimately define the specification for the new plant.

During this period, the use of ultrafiltration for buffer exchange, the separation of immunoglobulin Cohn pastes by filtration instead of centrifugation, the use of different filter aids and other chromatographic matrices, were investigated and developed.

The basic outline of the established process can be seen in the following schematic diagram (Figure 2).

The source material for albumin is Cohn Fraction II+III supernatant which is an offshoot from the immunoglobulin plant at Broadmeadows.

The Supernatant II+III is concentrated and then diafiltered against sodium acetate buffer using spiral wound, ultrafiltration cartridges with a nominal molecular weight cut-off of not less than 10 000. A euglobulin/lipoprotein precipitation step is then performed and the resultant precipitate filtered out by leaf filtration.
Next, two ion exchange steps are performed. Each of the ion exchange runs are performed on two 200 l columns, 1.2 m in diameter, with a 17.5 cm bed height, which are run in parallel (Figure 3).

The first ion exchange run uses an anion exchange medium, namely DEAE Sepharose™ Fast Flow. The albumin within the filtrate is bound to the medium. After loading, the column is washed with sodium acetate buffer to remove loosely bound proteins. The albumin is then eluted from the column with a second sodium acetate buffer (Figure 4).

The albumin eluate from the DEAE column is loaded directly onto the cation exchange medium, CM Sepharose Fast Flow. After loading, the CM Sepharose Fast Flow column is washed with sodium acetate buffer to remove loosely bound proteins. The albumin is then eluted with a sodium acetate buffer, and stored in large tanks in preparation for the next step (Figure 5).

Linear flow velocities range from 30 to 100 cm/h. After the third cycle the columns are regenerated with sodium hydroxide and re-equilibrated with buffer. At the end of a batch the columns are sanitized with, and stored in, sodium hydroxide. Height Equivalent to Theoretical Plate (HEPT) determinations are made on each of the columns between batches.

The albumin eluate from the CM Sepharose Fast Flow column is concentrated on spiral wound cartridges and then processed by gel filtration chromatography using Sephacryl™ S-200 High Resolution. The three columns, 1.0 m in diameter and each with a 30 cm bed height, are connected in series giving an effective bed height of 90 cm (Figure 6).

The first peak contains high molecular weight contaminants and albumin aggregates. The second peak contains dimeric albumin and part of the ascending side of the third peak. The total volume from these peaks is discarded. The third peak contains monomeric albumin which is collected for further processing.

The columns are sanitized between batches with sodium hydroxide. The eluted albumin is concentrated and diafiltered against Water For Injection, in preparation for final formulation.
To stabilize the albumin during pasteurization, 1.6 mm of sodium octanoate, or caprylic acid, per % gram of protein is added. On completion of formulation the product is sterile filtered into a bulk pasteurization tank and pasteurized for a minimum of 10 hours at 60 °C ± 0.5 °C (Figure 7).

The design of the bulk pasteurization tanks is such that all potential cold spots are eliminated. Heating is achieved by circulating heated glycol through the jacket. During validation, 64 temperature probes are used to ensure the elimination of cold spots, during routine processing 16 temperature probes are used. All temperature probes must be within set/defined limits prior to the start of, and during, the pasteurization run. Even the air space above the product in the tanks is agitated and monitored to ensure an even temperature distribution. The tanks are pressurized with heated sterile nitrogen during the run to minimize evaporation. No further pasteurization takes place after the bulk pasteurization. On the completion of the run, the albumin is hard piped to the sterile suite for dispensing.

Once dispensed the product is incubated at 31 °C for 16 days then 100% inspected, QC tested, labelled, packed and released for distribution (Figure 8).

After 4 years of operation, and processing of approximately 1 400 tonnes of plasma equivalent product through the albumin plant, some key characteristics about the plant can be established.

In terms of operation, the plant is highly predictable. This is due not only to the nature of the process, but also in part to the ease with which this process can be automated. The chromatographic plant demonstrates consistently reproducible high quality results. A batch has never been failed for endotoxin or PKA levels. Furthermore, the plant has even successfully re-processed Cohn albumin products which had previously failed pyrogens, PKA and particle release limits from a fully validated and approved process.

Other aspects of the chromatographic albumin plant to be considered are:

- Media replacement can be a significant cost; however, to date, we have experienced good media life up to 5 years.
- Cost of manufacture is significantly lower with a chromatographic plant, particularly when the total product released to market is considered. This is based on our experience of running both chromatographic and Cohn plants.
- The capital cost of constructing a chromatographic plant from a greenfield site is equivalent to a Cohn plant, but plant maintenance for the chromatographic plant is lower.
- Yields from this process currently average 24-26 grams per litre of plasma. Yields are calculated on actual plasma pooled compared with final containers out the door.
- Our studies demonstrate that increasing plant capacity is relatively straightforward, by increasing the size of the columns, or by adding extra columns. This is achieved without too much disruption to existing operations and is the classic modular approach.
- Our experience demonstrates the ease with which new products or plasma proteins can be isolated by adjoining other chromatographic columns off the main spine of the albumin production line. Our new chromatographically-derived intravenous immunoglobulin is a perfect example of this benefit. It is also our firm held belief that future plasma products will be derived by some form of chromatographic process.

The advantages of a chromatographic albumin plant can be summarized as follows.

- Purity at 99.5%
- Low aluminium content (10 PPB)
- Very low endotoxin
- Very low PKA
- Improved process control leading to improved process predictability and capability
- Ties in with future technology trends
In antiviral therapy some of the serious side-effects of nucleoside analogues may be reduced by coupling the drugs to carriers that are selectively taken up by the target cells. In this way anti-HIV agents can be delivered to T lymphocytes and macrophages thereby resulting in fewer side-effects. A series of modified albumins was studied in order to test whether they could be used as carrier molecules.

It was discovered that some (neo)-glycoproteins designed as drug carriers for anti-HIV agents, exhibited an intrinsic antiviral activity even without coupling the antiviral drug AZT to them (Table 1) (1). For example, mannosylated albumins with a high sugar substitution showed antiviral activity. At first sight, this indicated sugar specificity and a sugar substitution threshold for the intrinsic activity. However, it appeared that these mannosylated albumins also exhibit the most pronounced negative charge. Therefore the effect of a negative charge of the (neo)-glycoproteins on HIV replication was studied in vitro. The antiviral activity was shown to be related to an increased negative charge of the particular (neo)-glycoproteins. Succinylation of albumin alone appeared to suffice for conversion of the protein into a potent anti-HIV agent.

A way in which HIV can infect CD4+ cells is by fusion of HIV infected cells with uninfected CD4+ cells leading to the formation of multinucleated giant cells (syncytia). Syncytium formation is supposed to be one factor leading to T4 cell depletion and a potent inhibitor of this process might be of great therapeutic value. Succinylated human serum albumin (suc-HSA) inhibited this syncytium formation in the same concentration range as that affecting HIV replication (Table 1). From the time of the addition studies it was concluded that suc-HSA interferes with the virus/cell fusion process (2).

A major drawback in the development of antiviral proteins and monoclonal antibodies for use in AIDS therapy is the discrepancy between the sensitivity of laboratory HIV-1 variants to treatment and the sensitivity of primary isolates (HIV-strains isolated from patients).

### Table 1. Inhibitory effects of the modified albumins on HIV-1-induced cytopathogenicity and giant cell formation.

All data represent mean values for at least three separate experiments.

<table>
<thead>
<tr>
<th>Compound</th>
<th>CC50 a</th>
<th>IC50 b</th>
<th>Syncytium assay c</th>
</tr>
</thead>
<tbody>
<tr>
<td>HSA</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Man7-HSA</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Man38-HSA</td>
<td>&gt;500</td>
<td>96.0</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Man40-HSA</td>
<td>&gt;500</td>
<td>48.5</td>
<td>100.0</td>
</tr>
<tr>
<td>Fuc10-HSA</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Fuc25-HSA</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Glu5-HSA</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Glu25-HSA</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Gal5-HSA</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Gal32-HSA</td>
<td>&gt;500</td>
<td>&gt;500</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Suc-HSA</td>
<td>&gt;500</td>
<td>1.8</td>
<td>&gt;500</td>
</tr>
<tr>
<td>Suc-Man7-HSA</td>
<td>&gt;500</td>
<td>1.8</td>
<td>&gt;500</td>
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<td>Suc-Glu5-HSA</td>
<td>&gt;500</td>
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<td>2.0</td>
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<td>1.6</td>
<td>5.0</td>
</tr>
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<td>1.6</td>
<td>10.0</td>
</tr>
<tr>
<td>Suc-Gal32-HSA</td>
<td>&gt;500</td>
<td>9.5</td>
<td>20.0</td>
</tr>
<tr>
<td>Dextran sulfate (M, 5000)</td>
<td>&gt;500</td>
<td>0.6</td>
<td>28.0</td>
</tr>
</tbody>
</table>

a 50% cytotoxic concentration based on the reduction of the viability of mock-infected MT-4 cells
b 50% inhibitory concentration, based on the inhibition of HIV-1 induced cytopathogenicity in MT-4 cells
c 50% inhibitory concentration, based on the inhibition of syncytium formation upon co-culturing of persistently HIV-1 infected HUT-78 cells with MOLT-4 cells

Ref. (1)
Table 2 shows that the primary isolates, both syncytium inducing and non syncytium inducing, are sensitive to suc-HSA, although higher concentrations are needed compared to the HIVIIIIB-strain (laboratory strain) (3).

After the in vitro virus studies, preclinical studies in animals were done (4).

A toxicity study in the rat showed that the product is safe at doses as high as 400 mg/kg. These doses lead to plasma concentrations that are much higher than the inhibitory concentration needed for an antiviral effect as shown by the virus/cell studies.

In addition, a tolerance study in monkeys was done. As found in rats, multiple dosing of succinylated simian serum albumin (suc-SSA) in monkeys did not affect clinical parameters and blood count. After intravenous administration of a relatively low dose of 0.1 mg/kg, suc-SSA disappeared rapidly from the blood in a first-order kinetic pattern. The half-life was about 2 min, whereas at the highest dose of 3 mg/kg the corresponding apparent half-life was several hours, indicating a saturable elimination process. Also in the rat, similar saturation kinetics were observed. Importantly, suc-HSA was shown to distribute rapidly to the lymphoid tissue as indicated by an accumulation in the lymph. The efficient distribution to the lymphatic system is of particular interest for HIV therapy, taking into account that replication of HIV mainly takes place in the lymphoid system.

The efficacy and safety of suc-HSA in humans will be subsequently studied. At CLB, a pharmaceutical suc-HSA product has been manufactured that will be used for a pilot study in 10 to 20 AIDS patients that have become refractory to the classical drugs.

The product is synthesized as outlined in Figure 1. Albumin normally contains 60 lysine-residues with a positive charge. By succinylation these positive charges are turned into negative charges. After succinylation, formulation and lyophilization the freeze dried product, suc-HSA, is obtained. The process yield is around 90% at this scale. This product, synthesized as outlined in Figure 1, is biologically active and biochemically comparable to the suc-HSA used in the preceding experimental studies.

It can be concluded that suc-HSA is a promising new product with a potent anti-HIV activity. The installation of a pilot-scale production line (200 g scale) is now ongoing to produce material for a pilot study in humans to assess safety and efficacy.

### Table 2.

Characteristics of HIV-1 isolates and the effect of modified human serum albumin on virus replication.

<table>
<thead>
<tr>
<th>HIV-1 variant</th>
<th>SI capacity</th>
<th>Transmission to MT-2</th>
<th>Suc-HSA</th>
<th>IC50 (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV-1 IIIIB</td>
<td>+</td>
<td>+</td>
<td></td>
<td>3.0 ± 0.7</td>
</tr>
<tr>
<td>ACH-320.2A.1.2</td>
<td>+</td>
<td>+</td>
<td></td>
<td>263.3 ± 99.1</td>
</tr>
<tr>
<td>HIVams 16.2</td>
<td>-</td>
<td>-</td>
<td></td>
<td>39.7 ± 10.3</td>
</tr>
<tr>
<td>ACH-239.11</td>
<td>-</td>
<td>-</td>
<td></td>
<td>154.3 ± 104.4</td>
</tr>
<tr>
<td>HIVams 181</td>
<td>-</td>
<td>-</td>
<td></td>
<td>47.2 ± 8.99</td>
</tr>
</tbody>
</table>

a Syncytium induction in PBMCs  
b Replicative capacity in the MT-2 cell line  
c Mean concentration ± SE of modified HSA that caused a 50% reduction in virus titers Ref. (3).

References:
Development and production of coagulation factor concentrates: threats or opportunities?

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Chairman of session on coagulation factors

Coagulation factor deficiencies in humans have been well documented in the literature. However, their prevalence is low, in almost all cases, varying from 1:500 for homozygous Factor XI deficiency in the population of Askenazy Jews, or 1:12,000 for Factor VIII deficiency, to less than 1:1,000,000 for deficiencies of Factors II, V, X or XIII. In some cases a coagulation factor deficiency does not lead to clinical problems. This is true for deficiencies of the contact system (Factor XII, prekallikrein and HMWK). For other deficiencies, clinical symptoms can be severe, requiring regular transfusion of concentrates to treat or prevent life-threatening bleedings. This is the case for haemophilia A and B, but also in e.g. type III Von Willebrand’s disease.

Over the years we have seen major achievements in the development and production of coagulation factor concentrates, such as production of high-purity concentrates, but have also witnessed dramatic events, like the AIDS epidemic among haemophiliacs. In the first decades after 1950, the main goal was to develop preparations of Factors VIII and IX more concentrated than just plasma. The next era was spent making those preliminary concentrates more pure in order to achieve higher concentrations. With the increasing use of these high-purity concentrates, which were produced from large pools of plasma, something had to be about the viral risks. So, from the 1980s on, development work was dedicated to increasing the viral safety of these products. During the last decade, we have seen the advent of recombinant-Factor VIII and IX products, another major achievement. Traditionally, the plasma fractionation industry has mainly focused on Factor VIII and IX concentrates. However, for the more rare deficiencies the choice of concentrates for treatment is limited. For instance, for treating Von Willebrand’s disease and Factor XIII deficiency, only a few safe and effective concentrates are available.

Obvious threats include the transmission of viral disease, but there are other - others like thrombogenicity of Factor XI and prothrombin complex concentrates, product-related induction of inhibitors, and other adverse reactions that have occurred in the past. Improvements over the last decade have yielded much safer concentrates, but there still remains potential risks, such as shortages of product, and new problems may appear in the future, like nvCJD. For recombinant and transgenic products little is known about how the threats will manifest themselves. A shorter than normal half-life has been observed for transgenic antithrombin III and alpha-1-antitrypsin, and a lower than expected in vivo recovery for recombinant Factor IX. Looking on the brighter side, we know there is a good chance that remaining viral risks will be eliminated in the near future due to implementation of more effective, and double or even triple virus reducing methods. With the advent of ultra-high purity concentrates in large supply, alternative methods of administration may lead to less cumbersome ways of treatment. Also, modification of coagulation factors, either by genetic engineering or by more classical chemical approaches, may lead to a longer in vivo half-life or higher efficacy, or to therapeutic approaches that have a more sophisticated, coagulation modulating design than just substitution of a lacking component. Mass production by recombinant techniques has the potential, at least in theory, of providing large quantities of cheap concentrates. After all, it should not be forgotten that up till now only a minority of the coagulation-deficient patients among the world population receives adequate treatment.
The treatment of hemophilia using plasma-derived clotting factors has brought unquestioned benefit to patients. Concentrates developed in the 1960s permitted persons with hemophilia to receive home care and to have surgery when necessary, but with the risk of viral transmission. This risk was mitigated by the development of sensitive assays for hepatitis B and the hepatitis B vaccine. However, efforts to achieve higher degrees of purity were constrained by a lack of methods to purify further without losing the therapeutic activity of the clotting factors, which were thought to be highly labile.

The realization in the 1980s that the blood supply had been contaminated by the human immunodeficiency virus (HIV), with the result that many persons with hemophilia contracted AIDS, encouraged acceptance of the aggressive viral inactivation methods. These included dry heating, wet heating, betapropiolactone treatment and solvent detergent treatment.

The concept of affinity chromatography utilizing monoclonal antibody (MAb) ligands, with the capacity to bind only to the specific coagulation protein, was developed in the 1980s. It resulted in the introduction of coagulation products of a very high degree of purity such as the Factor VIII concentrate, Monoclate™ HT, licensed in 1987 by Armour Pharmaceutical. The advance in hemophilia treatment therapy can be measured by the reduction in the infusion volumes of fluid and protein load given to a patient requiring 1000 units of Factor VIII. A patient being treated with plasma alone would be given a litre of fluid with a protein load of 65 grams. The use of cryoprecipitate reduced the infusion volume to 500 ml and the protein load to 2.7 grams. Anti-hemophilic Factor (AHF) concentrates permitted the infusion volume to be reduced to 50 ml, and the protein load to 1.4 grams. The introduction of Monoclate HT was a major breakthrough, lowering the infusion volume to 10 ml and the protein load to 0.33 mg (excluding stabilizers), which is less than the protein load delivered with AHF concentrate by a factor of more than 4 000.

The use of immunoaffinity chromatography purified products and recombinant Factor IX products has enabled similar progress in developing products for hemophilia B therapy.

**Manufacture/purification of Monoclate-P, Factor VIII:C (human) pasteurized monoclonal antibody purified**

Monoclate-P™, a modification of Monoclate HT, with the addition of a pasteurization step, was licensed in 1989 by Armour Pharmaceutical Company.

Figure 1 shows the manufacturing steps for Monoclate-P and Figure 5 is a schematic of the purification process. The first and most vital step is the pasteurization of the crude AHF solution. The pasteurized AHF is passed through a column packed with resin to which MAb for Factor VIII·R (von Willebrand Factor) has been coupled. The Factor VIII molecule, consisting of Factor VIII·C (clotting activity) and VIII·R, is adsorbed onto the MAb. Non-bound protein and viral particles are washed out. Calcium chloride is used to dissociate VIII·C from VIII·R, which
remains bound to the antibody, and Factor VIII:C is collected in the eluate. Factor VIII:R is removed from the MAb with a solution of sodium thiocyanate, regenerating the immunoaffinity chromatography column. Finally, the isolated Factor VIII:C is further purified in an aminohexyl (AH) Sepharose™ column, formulated and freeze-dried.

The immunoaffinity chromatography process has been demonstrated to produce Factor VIII of very high purity. Data from several experiments showing a mean specific activity (SA) of 3712 units per mg have been presented.

The safety of Monoclate-P in terms of reduction of HIV titre is more than 16 logs. Pasteurization alone achieves 10.5 logs of this reduction, and processing an additional 6 logs.

To evaluate the efficacy of the purification process, model or marker viruses are used in place of viruses that cannot be cultured in vitro. Figure 3 shows four marker viruses plus HIV. Six to seven logs were reduced to undetectable levels in less than 10 hours of pasteurization at 60 °C.

Pasteurization is effective against lipid-enveloped and most of the non-enveloped viruses. Another method, the solvent detergent process, is effective against lipid-enveloped viruses only. As shown in Figure 4, 20 hours of treatment with the solvent detergent process did not inactivate EMC, whereas 10 hours of pasteurization inactivated 7 logs of EMC to undetectable levels.

Table 1 shows the effectiveness of the viral reduction processes used in the manufacture of Monoclate-P. Using pasteurization and MAb chromatography alone, HIV, bovine viral diarrhea (BVDV), pseudorabies, and polio viruses are reduced by >12.5, >9.2, >8.4 and >1.1 logs respectively. Parvovirus is reduced by 2.8 logs only since it is not inactivated by pasteurization. Immunoaffinity data for the other three viruses were taken from the first-generation Monoclate HT process. We believe that if the experiments are repeated with the current Monoclate P process, a similar log reduction will be achieved. Nevertheless, total log reduction presented for Sindbis, VSV, and Vaccinia is from pasteurization only; MAb chromatography reduction values have not been added.

Table 1. Monoclate-P total virus reduction (log_{10}) values.

<table>
<thead>
<tr>
<th>Processing Step</th>
<th>HIV</th>
<th>BVDV</th>
<th>PRV</th>
<th>Sindbis</th>
<th>VSV</th>
<th>Vaccinia</th>
<th>Polio</th>
<th>Parvo</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pasteurization</td>
<td>&gt;7.1</td>
<td>&gt;5.81</td>
<td>&gt;5.34</td>
<td>&gt;7.29</td>
<td>&gt;7.54</td>
<td>&gt;6.06</td>
<td>&gt;7.33</td>
<td>(0.52)</td>
</tr>
<tr>
<td>MAb chromatography</td>
<td>&gt;5.49</td>
<td>3.40</td>
<td>3.05</td>
<td>2.84*</td>
<td>1.23*</td>
<td>4.41*</td>
<td>3.73</td>
<td>2.8</td>
</tr>
<tr>
<td>AH-Sepharose chromatography</td>
<td>1.13</td>
<td>N.D.</td>
<td>(0.59)</td>
<td>1.8*</td>
<td>0.9*</td>
<td>N.D.</td>
<td>2.58</td>
<td>(0.95)</td>
</tr>
</tbody>
</table>

1 Total LRV >13.72 >9.2 >8.39 >7.29 >7.54 >6.06 >13.64 2.8

1 = The average virus reduction value is used in calculating total log reductions (LRV)
N.D. = Not determined
* = Data obtained for Monoclate HT

Table 2. Mononine (Marburg + QBI) total virus reduction (log_{10}) values.

<table>
<thead>
<tr>
<th>Processing Step</th>
<th>HIV</th>
<th>BVDV</th>
<th>PRV</th>
<th>Porcine Parvo</th>
<th>Canine</th>
<th>EMC</th>
<th>HAV</th>
<th>Sindbis</th>
<th>VSV</th>
<th>Vaccinia</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAb chromatography</td>
<td>&gt;5.8</td>
<td>&gt;5.8</td>
<td>&gt;8.1</td>
<td>3.7</td>
<td>4.3</td>
<td>&gt;6.0</td>
<td>&gt;3.9</td>
<td>3.3</td>
<td>&gt;2.8</td>
<td>&gt;7.2</td>
</tr>
<tr>
<td>YM-100 filtration</td>
<td>&gt;5.9</td>
<td>&gt;6.5</td>
<td>&gt;7.4</td>
<td>8.0</td>
<td>&gt;6.2</td>
<td>&gt;6.0</td>
<td>&gt;7.8</td>
<td>&gt;5.1</td>
<td>&gt;7.5</td>
<td>&gt;7.1</td>
</tr>
</tbody>
</table>

1 Total log_{10} reduction >11.7 >12.3 >15.5 >11.7 >10.5 >12.0 >11.7 >8.4 >10.3 >14.3 >10.9

1 = The average virus reduction value is used in calculating total log reductions
The manufacture and purification process for MononineTM, a Factor IX product of very high purity, is also shown in Figure 2 and 5. Cryo-poor plasma is processed to diethylaminoethyl (DEAE) concentrate. This is passed through a column packed with a resin to which MAb against Factor IX has been coupled. Factor IX is absorbed and impurities are washed out. Sodium thiocyanate (NaSCN) is used to elute Factor IX from the MAb. Incubation of Factor IX with NaSCN destroys any HIV that may be present. Two filtration processes follow. Diafiltration removes NaSCN, and viral-retentive ultrafiltration (YM100) removes molecules of $\geq 100,000$ daltons and viruses, but allows the Factor IX molecule (60,000 daltons) to pass through. Factor IX is then concentrated, passed through a chromatography column containing AH Sepharose to remove trace murine antibodies, formulated, and lyophilized. The resulting Mononine product has a SA of 250 U/mg protein, a level close to absolute purity. Western blot and SDS page electrophoresis analysis show that Mononine has virtually no Factor II, VII and X impurities. A negative Factor IXa clotting assay and a rabbit stasis model confirms non-thrombogenicity of Mononine. Absence of heparin in formulation assures that non-thrombogenicity assays are not compromised by false negatives.

Virus removal during MAb immunoaffinity chromatography of Monoclate-P and Mononine were achieved using the small-scale chromatography models to mimic manufacturing. Most of the virus was removed in the flowthrough and washes. Trace amounts close to the sensitivity of virus assay were detected in few eluates, in most cases the virus was undetectable in the eluate. Examples were presented for two viruses showing accountability of the spiked virus in different fractions, and the consistency of the process. We believe the MAb chromatography process to be quite robust due to the high specificity of antibody-antigen interaction. Experiments were, however, repeated with four viruses following the CPMP and ICH guidelines, and as anticipated, equivalent viral clearance numbers were obtained, confirming the validity of our earlier results.

Table 2 shows the composite of all virus validation data for Mononine. The combination of two virus removal steps produces results ranging from $>10.3$ to $>15.5$ log reduction for lipid-enveloped viruses, and $>8.4$ to $>12.0$ for non-lipid-enveloped viruses. Log reduction for both Porcine parvo and Canine parvo viruses, surrogates for human Parvo B-19, are effectively removed by this process. This is also confirmed by data showing that 76.7% of human patients treated with coagulation concentrates demonstrated B-19 transmission, with the exception of those patients treated with Mononine, who had no viral transmission.¹

Conclusion

Immunoaffinity chromatography using MAb, in combination with another virus inactivation step (pasteurization for Factor VIII and YM-100 filtration for Factor IX) results in the highest purity plasma-derived Factors VIII and IX concentrates. Both Monoclate-P and Mononine have a proven safety record from viral contamination.

Reference:
Immunogenicity of heat treated therapeutic plasma derived products

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Heating therapeutic plasma-derived products is a procedure commonly used to reduce or modify the risk of viral transmission. This procedure may cause denaturation of proteins leading to a reduction or total loss of functional activity. It is theoretically possible that new (neo)antigens may be generated as a result of this denaturation which could render the protein immunogenic in humans. An increase in the clinical incidence of inhibitor antibodies to Factor VIII (FVIII) has been attributed to the use of a pasteurized plasma-derived FVIII concentrate (1). However, to date there has been no direct link in the scientific literature of process related denaturation of proteins, neoantigen generation and immunogenicity.

The major focus of the neoantigen research on therapeutic proteins has been on FVIII concentrates because of the recognised concern of the inhibitor problem in this select group of patients. Up to 30% of patients treated by FVIII infusions produce antibodies that neutralise the procoagulation activity of the molecule. However, the major risk factors that have been identified in multicenter trials are race, family history and a FVIII gene defects, such as mutations, deletions and intron 22 inversion (2). In the subjects with FVIII gene mutation, deletions and intron 22 inversion (2), in vivo techniques. These studies were conducted to assess whether the heating procedure employed for viral inactivation during manufacture causes antigenic changes in the product. A panel of five murine monoclonal antibodies, directed to distinct epitopes on the FVIII molecule, were used to characterize the FVIII molecules in two severe dry heat treated FVIII concentrates. In this experiment, AHF(High Purity), an intermediate purity concentrate which has a good clinical record with regards to inhibitor formation, and Biostate™, a new high purity FVIII concentrate, were compared with a positive control, AHF(High Purity) which had been deliberately denatured. The positive control sample, obtained by inducing deliberate denaturation of FVIII by heating the concentrate in solution without stabilisers for 2 hours at 70 °C, was devoid of FVIII activity as assessed by the two-stage chromogenic assay. Figure 1 illustrates a typical binding curve generated by each monoclonal antibody to AHF(High Purity), Biostate and the positive control, AHF(High Purity) and Biostate showed comparable titration curves demonstrating that the epitopes recognised by the panel of monoclonal antibodies are identical in both AHF(High Purity) and Biostate. The titration curves obtained for positive control material indicated that the epitopes defined by the monoclonal antibodies were sensitive to heat induced denaturation of the FVIII molecule.

Similar epitope characterization studies have been performed to assess changes in the ATIII molecule over pasteurization. Commercially available polyclonal antibodies raised in rabbit and goat and a mouse monoclonal antibody to human ATIII were titrated on ATIII from Thrombotrol™-VF by ELISA (data not shown). Identical titration curve obtained showed that the pasteurization conditions employed in the manufacture of Thrombotrol-VF do not alter the reactivity of the antibodies. This confirms that the immunodominant epitopes of the ATIII molecule are unaltered by pasteurization.

Studies aimed at revealing possible differences in the immunological properties between AHF(High Purity) and Biostate were also performed in rabbits according to the method published by Ronneberger (5). Rabbits were immunized intravenously with either AHF(High Purity) or Biostate. The presence of anti-FVIII antibodies in the sera of rabbits immunized with Biostate that were non-reactive against AHF(High Purity) was used as an indication of the generation of new epitopes in the Biostate manufacturing process. Titration curves observed from all anti-AHF(High Purity) immunoglobulin (rabbits a-e) were
Similar Biostate. of new epitopes on the FVIII molecules of new antibodies and therefore the absence are identical (Figure 4). The absorption of molecules of AHF (High Purity) and Biostate immunodominant epitopes on FVIII the alternate antigen suggests that the Absorption of reactivity of immune Ig with all anti-Biostate immunoglobulin (rabbits f-j) were similar when titrated on AHF (High Purity) and Biostate FVIII coated plates, see Figure 3.

In addition, titration curves observed from all anti-Biostate immunoglobulin (rabbits f-j) were similar when titrated on AHF (High Purity) and Biostate coated plates, see Figure 2.

Absorption of reactivity of immune Ig with the alternate antigen suggests that the immunodominant epitopes on FVIII molecules of AHF (High Purity) and Biostate are identical (Figure 4). The absorption of reactivity further suggests the absence of new antibodies and therefore the absence of new epitopes on the FVIII molecules of Biostate.

Similar in vivo studies were performed with Thrombotrol-VF. Rabbits were administered either Thrombotrol-VF final product or Thrombotrol-VF prepasteurized intermediate subcutaneously with adjuvant. The use of adjuvant in this study was necessary because ATIII in Thrombotrol-VF was found to be a poor immunogen. The lack of immunogenicity of native Thrombotrol-VF is not surprising, given the highly conserved nature of the molecule across species. ATIII from sheep, mouse and rabbit have been cloned and sequenced. When these molecules are compared to the human molecule, the sheep and mouse share 89% and the rabbit 84% identity to the human ATIII. Given the high homology which exists between the human, mouse and rabbit molecule one can conclude the failure to elicit and immune response against both Thrombotrol-VF final product and Thrombotrol-VF pre-pasteurized intermediate in these species confirms that the pasteurization process does not alter the immunogenicity of the ATIII molecule.

There have been no incidence of inhibitor antibody development in clinical studies on patients exposed to multiple doses of Biostate. All patients treated with product recorded less than 0.5 Bethesda units and a repeat pharmacokinetic profile obtained was similar to the initial pharmacokinetic study with respect to FVIII half-life and in vivo recovery. Serum samples from patients with haemophilia B who were exposed to multiple doses of MonoFIX™, in which ATIII is present as an excipient, have been tested for anti-ATIII antibodies by ELISA and ATIII antigen levels by Laurell immuno-electrophoresis. There were no detectable anti-ATIII antibodies in the sera of these patients and all patients recorded normal levels of ATIII.

In conclusion, the immunological properties of Biostate, a new high purity severe dry heat treated FVIII concentrate and Thrombotrol-VF, a pasteurized human ATIII concentrate have been characterized in vitro, in animals and in clinical studies. The results reported provide a substantial database on the potential of these two products to cause immunogenicity. It is therefore possible to suggest that the Biostate and Thrombotrol-VF manufacturing processes do not induce the formation of new epitopes and the heat treatments employed in their manufacture do not render the final concentrates immunogenic.

References
Today the need for safe and high quality plasma products is clearly evident and is reflected in the demands from both patient groups and authorities. To meet these growing demands, the plasma fractionation industry must continuously develop and improve the fractionation processes. The fractionation processes of HemaSure Denmark A/S, Denmark, reported here, describes how the company met this challenge by developing an alternative method to the traditional Cohn method for fractionating plasma.

From the very start of the company in the mid-1970s, the idea was to develop fractionation processes which use gentle conditions and operate at room temperature with short processing times that result in plasma products of consistently high quality and consistently high yield. At the same time there has been much focus on producing efficient therapeutic plasma products with a high degree of safety against pathogen transmission. This has been addressed by implementing inactivation steps for both lipid and non-lipid enveloped viruses (double virus inactivation).

Polyethylene glycol (PEG) and caprylic acid were chosen as precipitation agents in order to avoid the inherent denaturation in the method developed by Cohn et al (1) to allow the processes to be operated at room temperature with very short processing time. This reduces costs for handling and cooling of the product. PEG and caprylic acid are used in the fractionation processes for albumin and intravenous immunoglobulin (IVIG). Chromatography has been introduced in parts of the processes to achieve native products of high purity and high yield. The well-known cryoprecipitation step has been exchanged for an initial high capacity gel filtration step to isolate Factor VIII (Nordiate™) (Figure 1) and the von Willebrand Factor (vWF) directly from plasma in significantly higher yields and of higher quality than were previously possible (2, 3). The rest of the manufacturing process for Factor VIII and vWF is also based solely on chromatography.

Since its implementation in 1993 in production scale, the high capacity gel filtration step has given a step yield of 60-70% of coagulation factor VIII (FVIII:C), which is superior to the 40-50% step yield of the cryoprecipitation step. The yield from the whole process is 200 International Units (IU) FVIII:C/litre plasma, with a specific activity of 300 IU FVIII:C/mg protein, which makes Nordiate a high purity Factor VIII product.

Nordiate has been shown to contain high amounts of vWF, inclusive of the haemostatically important high molecular weight vWF, but not in quantities to be therapeutically effective in patients suffering from von Willebrand’s disease.

Through further development of the existing Nordiate process the company succeeded in improving the yield of vWF in the final product, resulting in a Factor VIII-enriched vWF product (more than 95% pure), in contrast to Nordiate which is a vWF enriched Factor VIII product. The new vWF is a slight modification of the well-established Nordiate process. In fact the vWF product is manufactured in the very same production plant as Nordiate. It has therefore been relatively easy to introduce the new process, avoiding new investments and comprehensive staff training, etc.
From a clinical point of view, this newly developed vWF looks very promising as it 1) seems haemostatically active, 2) has a high purity, 3) has a small injection volume, and 4) has a high degree of safety with regard to viral infection as it has been subjected to two independent virus inactivation steps.

The double-virus inactivation procedure for Nordiate and the vWF-product consists of an in-process solvent/detergent step to inactivate lipid enveloped viruses, and severe heat treatment (80 °C, 72 hours) of the lyophilized products in the final vial to inactivate non-lipid enveloped viruses.

The gel filtration step separates the albumin/globulin-fraction from the FVIII/vWF-fraction. Further purification of albumin and intravenous immunoglobulin is based on PEG and caprylic acid precipitations and ion exchange. The in-process treatment of the plasma fractions with non-ionized caprylic acid has been validated and shown to be specific inactivation steps for lipid enveloped viruses.

Figure 2 shows the manufacturing process for Albumin HemaSure which has to include two individual virus inactivation steps, i.e. incubation with caprylic acid (heat precipitation) and pasteurization of the final vials (60 °C, 10 hours). This gives a final product with a purity of more than 99% and with less than 0.3% of polymers and aggregates.

A native high purity intravenous immunoglobulin (Nordimmun™), with all IgG subclasses present in proportions similar to those found in plasma, has been separated by introducing an efficient ion exchange step after the initial PEG and caprylic acid precipitation steps (Figure 3).

The purified IgG is left in the column flow-through, while impurities, including IgA, is bound to the column. Nordimmun has a purity of more than 99% and contains less than 1% of polymers/aggregates (before addition of albumin); Pasteurization (60 °C, 10 hours) has been added as the second virus inactivation step before final formulation and lyophilization. A liquid formulation of Nordimmun (without lyophilization) has been developed and shown to be stable at 25 °C/60%RH for two years.

**Conclusion**

The development of an alternative fractionation strategy produced safe and high quality plasma products which meet the demands from both patient groups and regulatory authorities.

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**References**

Intravenous Immune Globulin G (IGIV) is used to treat a broad spectrum of immunodeficiency syndromes including HIV indications, chronic fatigue syndrome, idiopathic thrombocytopenic purpura, as well as in bone marrow transplantation. Demand for IGIV has escalated over the past 20 years and currently exceeds availability.

In an attempt to redress the imbalance between supply and demand for IgG, Bayer Corporation, Clayton, NC, USA, a leading producer of IgG in the US, has been investigating means of improving product recovery. The company has recently developed a method based on chromatography which gave a product with the same high purity as its proprietary IVIG-SD, but with a 50% increase in yield. During development of the method, the company screened 42 different chromatographic media; best results were achieved with Sepharose™ Fast Flow ion exchangers.

There were several considerations in developing this new method. Bayer’s current proprietary process purifies IgG from human plasma intermediates (Cohn Fraction II & III pastes) and these were to be the starting materials for the new method. It was also important for the process to integrate virus inactivation into the purification scheme. And finally, the new process had to be able to process large quantities, more than 200 kg paste per batch.

Virus removal and inactivation are incorporated into the methods for dissolving the pastes. By adding the sodium salt of caprylate to the IgG suspension, lipoproteins are removed and enveloped viruses are inactivated. Precipitates are removed by filtration. The IgG-containing solution is at a low ionic strength, which is beneficial for chromatography since no additional treatment is necessary. Compatibility with the caprylate conditions was thus a condition for screened media.

The chromatography step was designed for IgG flow-through and capture of impurities. Capturing IgG would require a high-salt elution buffer to elute the protein. The combination of high salt and high IgG concentration is believed to promote aggregation of IgG, which can lead to anti-complimentary activity and subsequent adverse reactions in patients when administered intravenously.

Media screening was performed on columns with an internal diameter of 11 mm using standardised conditions for bed heights, pH, flow rates, conductivity and loading volumes. The different media were compared on the basis of their resulting product purity and yield. With the media selection procedure completed, the combination of column and medium was optimized. After the process was defined at lab-scale, the process was scaled-up in four steps for the production of clinical supply lots.

The chromatographic scheme comprised anion exchange followed by cation exchange: The first column contained a Sepharose™ Fast Flow strong anion exchanger, which bound all detectable albumin, most of the IgA and some IgM, as well as the majority of the caprylate. The second column contained a Sepharose Fast Flow weak anion exchanger and bound the remaining IgM and the rest of the contaminants. In addition, Bayer linked the two columns in series, which meant they could use smaller columns. Following chromatography the IgG solution is diafiltered, concentrated, and compounded for sterile dispensing.

Bayer reports that the development of this chromatography-based method represents significant improvement over alcohol fractionation methods. Product purity and yield are high, neither a salt elution step nor a special step to remove the caprylate is needed, and column size is reduced. The method works with other combinations of strong and weak anion exchangers. The Sepharose Fast Flow resins were chosen because they had the best combination of performance and reproducibility.

(This article was previously published in Downstream 30)
Chromatographic purification of immunoglobulins

Joseph Bertolini, CSL Bioplasma, Melbourne, Australia

In 1994, CSL embarked on a project to develop a process that would improve the yield of immunoglobulin production and add to its viral safety.

The impetus came from the recognition that in addition to the established role of intravenous immunoglobulins in the treatment of agamaglobulinaemia, idiopathic thrombocytopenia purpura (ITP) and Kawasaki Syndrome, there was a growing demand for the product in many other autoimmune conditions, such as demyelinating polyradiculoneuritis, and Guillain-Barre Syndrome.

With respect to viral safety, customers and regulatory authorities were demanding enhanced assurance.

Figure 1 presents a flow chart of the manufacturing process devised for immunoglobulins using the chromatographic approach.

The plasma was processed to Supernatant I then delipidated. Following adjustment to pH 5.2, which resulted in the isoelectric precipitation of some proteins, the solution was clarified and loaded onto a DEAE Sepharose™ Fast Flow column. The recovered crude IgG was passed through a second column (MacroPrep™ HQ) to generate the pure immunoglobulin fraction. Following concentration and diafiltration, the immunoglobulins were formulated with sorbitol and pasteurized. The solution was then diafiltered to remove the sorbitol and the immunoglobulin formulated into final product of 6% protein in 10% maltose at pH 4.25. Under these conditions, there was no effect on immunoglobulin integrity, as shown by the absence of any aggregate formation. The second viral inactivation step involved incubation of the dispensed product formulated at pH 4.25, at 27 °C for 14 days.

Figure 2 presents the kinetics of viral inactivation achieved using these two inactivation procedures. Pasteurization resulted in 5-6 log removal of pseudorabies virus (PRV), encephalomyocarditis virus (EMC), bovine viral diarrhoeal virus (BVDV) and human immunodeficiency virus (HIV). Even EMC and PRV non-enveloped coated viruses are satisfactorily inactivated. Low pH incubation resulted in greater than a 5 log reduction of Sinbis, BVDV, HIV. These steps therefore result in significant reduction of both enveloped and non-enveloped viruses.
The final product has been subjected to extensive characterization. Some key results are summarized in Table I. Tests used include those required by the British Pharmacopoeia (B.P.), which form the basis for release testing and other ancillary tests designed to further establish the integrity and purity of the product. The product is stable at 2 to 8 °C for over 2 years.

Intragam™ P underwent successful clinical assessment for the treatment of primary immunodeficiency and idiopathic thrombocytopenic purpura. A half-life of 39.7 days was observed. This product is now registered in Australia.

### Table 1. Key features of Intragam P.

<table>
<thead>
<tr>
<th>Test Name</th>
<th>Limit</th>
<th>Intragam</th>
<th>Intragam P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha-2-Macroglobulin</td>
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</tr>
<tr>
<td>Apolipoprotein A1</td>
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<td>&lt;0.002</td>
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</tr>
<tr>
<td>Albumin</td>
<td>(mg/ml) 0.007 (0.018-0.155)</td>
<td>&lt;0.03</td>
<td>&lt;0.03</td>
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<td>Kallikrein</td>
<td>(% of standard) 118 (28-192)</td>
<td>47-138</td>
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<tr>
<td>Plasminogen</td>
<td>(µg/ml) 0.17 (&lt;0.1-0.24)</td>
<td>&lt;0.19</td>
<td>&lt;0.19</td>
</tr>
<tr>
<td>IgG subclasses</td>
<td>IgG1 (%) 62 (57-66)</td>
<td>55.5-60.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG2 (%) 33 (30-36)</td>
<td>35.1-40.3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG3 (%) 2.9 (2.3-4.9)</td>
<td>2.2-3.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>IgG4 (%) 1.1 (1.0-1.5)</td>
<td>4.3-4.8</td>
<td></td>
</tr>
<tr>
<td>IgA (RID)</td>
<td>(mg/ml) 0.31 (0.061-0.52)</td>
<td>&lt;0.018</td>
<td>&lt;0.018</td>
</tr>
<tr>
<td>IgM (RID)</td>
<td>(mg/ml) 0.08 (0.014-0.11)</td>
<td>&lt;0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>Freedom from ACA</td>
<td>≤10CH50 per mg of Immunoglobulin</td>
<td>&lt;2-4</td>
<td>2</td>
</tr>
<tr>
<td>Prekallikrein Activator PKA</td>
<td>≤28.6 IU/ml 3.7 (1.0-23.8)</td>
<td>&lt;1.0</td>
<td></td>
</tr>
<tr>
<td>Protein Composition B</td>
<td>IgG Monomer M 94.7 (91.9-98.3)</td>
<td>93.4-96.9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>and Dimer ≥90.0 0.1 (0-0.4)</td>
<td>0-0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Aggregates ≤3.0 0.1 (0-0.4)</td>
<td>0-0.1</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 2.** Viral inactivation of Intragam P.
Intravenous immunoglobulins (IVIGs) are the fastest growing market segment of the blood products industry with an ever increasing range of clinical applications. Although lyophilized IVIGs are well established and have been available for many years, the market is now demanding the development of stable liquid formulations.

Presentation of proteins in liquid format is a much more severe test of product stability than in the lyophilized state. An IVIG formulation must include stabilizers which minimize aggregation and other physicochemical changes during storage; such changes may compromise Fc functionality and contribute to undesirable side effects. The product must also be free of contaminants, such as proteases which may degrade the IgG during liquid storage, and other impurities, such as PKA (prekallikrein activator), which may cause hypotensive effects.

Formulation development of the liquid product was based largely on monitoring four key markers during short term accelerated stability studies:

(i) anti-complementary activity (adverse changes in the IgG molecule may lead to spontaneous complement activation which has been linked to serious adverse reactions in early products);

(ii) Fc function by Rubella SRH (reflecting the functional integrity of the IgG molecule);

(iii) molecular size distribution, to provide information on aggregation and fragmentation of the IgG preparation;

(iv) PKA activity, a potent hypotensive agent which may be a trace contaminant in such preparations or may be generated during storage.

Accelerated stability testing of a range of sugar, sugar alcohol and amino acid formulation variants was performed to select for optimal stability in liquid format. The chosen formulation, containing albumin and sucrose as the principle stabilizers, provided good stability and was similar to BPL’s lyophilized sister product, Vigam™-S; thus both lyophilized and liquid IVIG products could be made using a common manufacturing process with only minor differences at the terminal processing stages. The most significant revision to the liquid formulation is the lower pH, which is important for microbiological control, including inactivation of both lipid-enveloped and non-enveloped viruses.

Extensive in vitro testing was performed to characterize the new liquid IVIG product [Vigam™ Liquid] and ensure full regulatory compliance. Product testing fell into four main categories: control of the source plasma (virus marker testing, size of start pool, etc); control of the IgG ‘active ingredient’ (in terms of aggregate/monomer content, Fc function, subclass composition, antibody profile etc.); control of product purity (gammaglobulin purity and identification/quantification of trace contaminants such as IgA, IgM, PKA, anti-A/B haemagglutinins, pyrogens and SD reagents); control of product formulation including excipient levels, protein concentration, pH and osmolality.
The liquid product readily meets all of these requirements: the data indicate it is a high purity preparation with normal Fc function and very low levels of contaminating proteins and other impurities (Table 1).

The Vigam manufacturing process for both lyophilized and liquid IVIG products utilizes cold ethanol fractionation and chromatography (Figure 1), which also provide some virus-reducing capability. The principal purification step downstream of the main IgG intermediate, Fraction II, is batch adsorption with DEAE Sephadex™. Conditions are selected where only impurities such as IgA, IgM and PKA are bound - IgG passes through the ion exchanger without binding. The purity of the preparation after this stage is ~100% gammaglobulin. Solvent-detergent (SD) treatment involving incubation at 37 °C for 6 hours with 1% Polysorbate 80 and 0.3% TNBP is incorporated as a specific virus inactivation (VI) step. This well established and trusted VI technology is highly effective against lipid enveloped viruses such as HCV, HBV and HIV (see Table 2). Clearance of SD chemicals is achieved in two steps involving a crude soybean oil extraction followed by binding of the IgG to the cation exchanger, CM Sepharose™ Fast Flow, and removal of residual SD chemicals by extensive washing of the bound IgG. An additional feature of Vigam Liquid is the enhanced inactivation of viruses, including various non-enveloped viruses, during terminal incubation at low pH (Table 2).

An animal model, which closely simulated clinical use, was used to confirm the lack of cardiovascular effects prior to human volunteer and patient trials. Subsequently, the product has been shown to be well tolerated and efficacious in the clinical situation.

The licensed product has good stability at both 4 °C and 25 °C and meets or exceeds the European Pharmacopoeia monograph specification for intravenous human normal immunoglobulin. Vigam Liquid is a new generation liquid IVIG product which readily satisfies the current market demands for a product which is clinically well tolerated and convenient to use.

### Table 1. Vigam Liquid specification.

<table>
<thead>
<tr>
<th>Control of active ingredient</th>
<th>Compliance (Ph Eur/BPL)</th>
<th>Limits</th>
<th>Vigam Liquid</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aggregate (%)</td>
<td>Ph Eur</td>
<td>NGT 3</td>
<td>0.3*</td>
</tr>
<tr>
<td>Monomer/dimer (%)</td>
<td>Ph Eur</td>
<td>NLT 90</td>
<td>99.4*</td>
</tr>
<tr>
<td>Purity (% Gammaglobulin)</td>
<td>Ph Eur</td>
<td>NLT 95</td>
<td>100*</td>
</tr>
<tr>
<td>Anti-complementary activity (CH50/mg IgG)</td>
<td>Ph Eur</td>
<td>NGT 1.0</td>
<td>0.5</td>
</tr>
<tr>
<td>Fc function (Rubella SRH, iu/mg)</td>
<td>BPL</td>
<td>NLT 15</td>
<td>37</td>
</tr>
<tr>
<td>Subclass composition (% IgG 1: 2: 3: 4)</td>
<td>Ph Eur</td>
<td>as Normal plasma</td>
<td>61: 31: 7: 1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Control of Impurities:</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>PKA (iu/ml)</td>
<td>Ph Eur</td>
<td>NGT 35</td>
<td>&lt;2</td>
</tr>
<tr>
<td>Anti-A/B haemagglutinins</td>
<td>Ph Eur</td>
<td>NGT 1/64</td>
<td>1/2 - 1/16</td>
</tr>
<tr>
<td>Pyrogens (°C)</td>
<td>Ph Eur</td>
<td>Complies</td>
<td>0.5</td>
</tr>
<tr>
<td>IgA (% total protein)</td>
<td>BPL</td>
<td>NGT 0.02</td>
<td>&lt;0.02</td>
</tr>
<tr>
<td>IgM (% total protein)</td>
<td>Not specified</td>
<td>&lt;0.01</td>
<td></td>
</tr>
<tr>
<td>IgE (% total protein)</td>
<td>Not specified</td>
<td>Not specified</td>
<td>&lt;0.0002</td>
</tr>
<tr>
<td>TNBP (mg/l)</td>
<td>BPL</td>
<td>NGT 1</td>
<td>&lt;0.2</td>
</tr>
<tr>
<td>Polysorbate 80 (mg/l)IBPL</td>
<td>NGT 20</td>
<td>-10</td>
<td></td>
</tr>
</tbody>
</table>

*Prior to formulation with albumin
Ph Eur = European Pharmacopoeia
NGT = not greater than
NLT = not less than

### Table 2. Vigam Liquid: virus validation summary.

#### Enveloped virus

<table>
<thead>
<tr>
<th>Virus inactivation (log reduction value)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIV</td>
</tr>
<tr>
<td>PSR</td>
</tr>
<tr>
<td>Sindbis</td>
</tr>
<tr>
<td>HSV-1</td>
</tr>
<tr>
<td>SFV</td>
</tr>
<tr>
<td>VSV</td>
</tr>
<tr>
<td>Vaccinia</td>
</tr>
<tr>
<td>Process stage:</td>
</tr>
<tr>
<td>Solvent/Detergent (6 hr, 37 °C)</td>
</tr>
<tr>
<td>CM Sepharose FF (pH 4 + clearance)</td>
</tr>
<tr>
<td>Terminal low pH incubation</td>
</tr>
<tr>
<td>Total</td>
</tr>
<tr>
<td>Lipid enveloped viruses</td>
</tr>
<tr>
<td>HIV: Human Immunodeficiency Virus</td>
</tr>
<tr>
<td>PSR: Pseudorabies</td>
</tr>
<tr>
<td>Sindbis: Model for HCV</td>
</tr>
<tr>
<td>HSV-1: Herpes Simplex Virus-1</td>
</tr>
<tr>
<td>SFV: Semliki Forest Virus</td>
</tr>
<tr>
<td>VSV: Vesicular Stomatitis Virus</td>
</tr>
<tr>
<td>Vaccinia</td>
</tr>
<tr>
<td>Non-enveloped virus</td>
</tr>
<tr>
<td>EMC: Encephalomyocarditis Virus</td>
</tr>
<tr>
<td>BPV: Bovine Parvovirus</td>
</tr>
<tr>
<td>SV-40</td>
</tr>
</tbody>
</table>

#### Non-enveloped virus

<table>
<thead>
<tr>
<th>Terminal low pH incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>EMC</td>
</tr>
<tr>
<td>BPV</td>
</tr>
<tr>
<td>SV-40</td>
</tr>
<tr>
<td>Polio</td>
</tr>
</tbody>
</table>
Albumex™ is a chromatographically purified pasteurized albumin product derived from the supernatant II+III of the Cohn process. A purity of >98% and a monomer content of >99% is achieved by sequential ion exchange steps using DEAE Sepharose™ Fast Flow anion exchange and CM Sepharose Fast Flow cation exchange followed by a Sephacryl™ S-200 High Resolution gel filtration. Albumex 2VI is a recently developed chromatographic process which is derived from fibrinogen depleted plasma rather than the Supernatant II+III. It incorporates a low pH/caprylate virus inactivation step in addition to pasteurization.

The chromatographic purification of plasma proteins affords opportunities for virus removal which can contribute to the enhancement of the viral safety of the final product. The CPMP/BWP/268/95 Guidelines in Part 6.3 address virus removal through partitioning and state that "if a partitioning process gives a reproducible reduction of virus load and ... manufacturing parameters are properly defined and controlled ..... it could fit the criteria of an effective step." To comply with these guidelines, careful attention to scale-down and identification of parameters which may affect partitioning is of utmost importance. In particular, if parameters which are critical to the purification of the target protein are identified and worst case scenarios are performed reproducibly, demonstration of efficacy of viral clearance can be achieved.

Virus removal studies were performed on the ion exchange and gel filtration columns at a scale-down of 1 in 500, and 1 in 1500, respectively, at a high flow rate and a high protein concentration, which were the conditions considered to be least favourable for resolution of proteins, see Table 1.

The physicochemical parameters of the laboratory validation and production runs of DEAE and CM Sepharose Fast Flow and Sephacryl S-200 High Resolution, in the Albumex and Albumex 2VI process, demonstrated excellent scale-down. Similar albumin purity, >99%, was achieved following ion exchange, and a similar monomer content of >99% after gel filtration.

The influence of specific antibodies on partitioning of virus was highlighted in hepatitis A virus (HAV) spiking studies on the Albumex and Albumex 2VI processes. In the Albumex process, a 5.1 log reduction of HAV infectivity away from the albumin peak was observed in the IgG depleted Supernatant II-III through the DEAE Sepharose Fast Flow ion exchange column. A further 1.2 logs were removed through the CM Sepharose column, see Table 2.
In the Albumex 2VI process, where the partitioning of the HAV antigen-antibody complex was traced by PCR, IgG present in the pre-DEAE feed solution reduces the infectivity of the spiked HAV to below detectable levels. A reduction of only 1.4 logs of infectivity equivalent was noted through the DEAE Sepharose Fast Flow column, but the HAV RNA was reduced to below detectable levels in the CM Sepharose Fast Flow eluate albumin fraction, see Table 1, Figure 1. The Sephacryl S-200 High Resolution step is equivalent for both processes and greater than 4.5 logs of HAV partitioned away from the albumin fraction.

Canine parvovirus clearance through DEAE Sepharose and CM Sepharose Fast Flow was similar in both processes, which was as expected given that specific antibodies would not be present. Studies on hepatitis C virus (HCV), bovine viral diarrhoea virus (BVDV - a model for HCV) and poliovirus type 1 (PV1) through the Albumex process demonstrated greater clearance for HCV than for the model virus. PV-1 gave a clearance of only 1.6 logs through both media which was in contrast to HAV, another member of the Picornaviridae.

It is interesting to note that during anion exchange, viruses with isoelectric points greater than the pH 5.2 of the loading buffer would be expected to pass through in the wash. The fact that this does not happen indicates that factors other than isoelectric points, such as hydrophobicity, surface charge distribution, and perhaps even size may play a role, particularly in low ionic strength conditions. Similarly, with the CM Sepharose Fast Flow column, it would be expected that viruses with isoelectric points above 5.5, the pH of the elution buffer, would remain on the column. As in the DEAE Sepharose Fast Flow step, predicting binding by pI is not tenable.
Up to 1998, the solvent/detergent (SD) treatment was the only specific process step in CLB’s process for prothrombin complex concentrate to inactivate blood-borne viruses like HIV, and hepatitis B and C viruses. Validation of the other process steps for their virus inactivating or removing capacity showed no additional contribution to the viral safety of the product. As European guidelines for achieving viral safety of plasma derivatives advocate the implementation of two effective steps, of which at least one also deals with non-enveloped viruses, a number of virus reducing methods were evaluated for their potential to achieve this goal for this particular product.

Pasteurization, in the presence of protein stabilizers like sugars and amino acids, was not successful, as infectivity of canine parvovirus (CPV) was not reduced at all. Heat treatment of lyophilized product (72 hours 80 °C, 20 hours 90 °C or 10 hours 100 °C) yielded >4 logs reduction of CPV infectivity. However, upon high performance size exclusion chromatography (HPSEC) analysis, aggregation of proteins was apparent, accompanied by losses of coagulation factor activity by about 30%.

Nanofiltration was then evaluated as third option. In order to deal effectively with small non-enveloped viruses like hepatitis A virus (HAV) and parvovirus B19, filtration through a membrane with a pore size of 15 nm was considered to be the only sensible way. Initial experiments however resulted in almost instantaneous clogging of the nanofilter due to the presence of contaminating proteins of high molecular weight, such as inter-alpha-trypsin inhibitor, immunoglobulin M and fibronectin. Of several options for separating these contaminants from the coagulation factor proteins of interest, we tried sieving filtration as the method of choice. An ultrafiltration membrane with a cut-off of 200 kD proved to be successful at laboratory scale. Filtration through a 15 nm virus removing filter (Planova 15N, Asahi) was feasible, while recovery of coagulation factor proteins was better than 90%.

During scaling up of the process several problems needed to be solved, for instance low product recovery and unsolved clogging of the nanofilter. By decreasing the cut-off of the prefilter to 150 kD and increasing the filter surface area, a routine production setting was established in which 40 liters of product solution (equivalent to 360,000 IU of Factor IX and 200 gram of protein) are now filtered per m² of nanofilter. Product recovery is 80-85% in the prefiltering step and 90-95% in the nanofiltration procedure, resulting in an overall yield of about 75%. The loss is due to incomplete sieving filtration and other mechanical losses, rather than denaturation. In characterizing the product all essential parameters, including both in vitro and in vivo thrombogenicity, appeared to be unchanged. The only exception to this is the protein content, which is decreased by 30-40% resulting in an increase of the specific activity of coagulation factor IX from 1.0 to 1.6 IU/mg. As is shown in Figure 1, this loss in protein is caused by the removal of high molecular weight constituents.

A study of the robustness of the nanofiltration step using again CPV as a worst-case virus revealed that the use of a single nanofilter resulted in a reduction of infectivity by 3.8 to 4.3 logs. This result implies that some infectivity was still detectable in the nanofiltrate. However, applying two nanofilters in series resulted in the virtual absence of infectivity in the filtrate, even when a larger test volume was tested in the infectivity assay. The total reduction factor for CPV was better than 6.0 logs. A similar figure was found for HAV, which was already obtained using a single nanofilter.

In a separate study, alternatives to the Planova 15N nanofiltration was an effective and robust virus removing step, capable of dealing efficiently with small non-enveloped viruses. The procedure is mild for proteins since no denaturation or other product changes could be detected.

We conclude that double Planova 15N nanofiltration is an effective and robust virus removing step, capable of dealing efficiently with small non-enveloped viruses. The procedure is mild for proteins since no denaturation or other product changes could be detected.

Table 1. Viral reduction data for the Cofact process.

<table>
<thead>
<tr>
<th>Process step</th>
<th>HIV</th>
<th>BVDV</th>
<th>PSR</th>
<th>CPV</th>
<th>EMC</th>
<th>HAV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cryoprecipitation</td>
<td>0.2</td>
<td>-0.4</td>
<td>0.4</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>SD-treatment</td>
<td>≥6.1</td>
<td>≥5.2</td>
<td>≥4.1</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>2nd DEAE-adsorption</td>
<td>1.0</td>
<td>n.d.</td>
<td>1.1</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
<tr>
<td>Nanofiltration</td>
<td>≥7.0</td>
<td>≥5.9</td>
<td>≥6.2</td>
<td>≥5.9</td>
<td>≥7.3</td>
<td>≥5.9</td>
</tr>
<tr>
<td>Total reduction</td>
<td>&gt;13.1</td>
<td>&gt;12.1</td>
<td>&gt;11.4</td>
<td>&gt;5.9</td>
<td>&gt;7.3</td>
<td>&gt;5.9</td>
</tr>
</tbody>
</table>

n.d.: not determined
n.a.: not applicable

Figure 1. HPSEC analysis of prothrombin complex before prefiltration, after prefiltration and after nanofiltration.
Pentose Pharmaceuticals (now VITEX after the merger in November 1999) has developed a novel technology for selective inactivation of viruses in plasma derivatives, biopharmaceuticals and transfusion blood components whilst preserving their structural integrity and functional utility. The technology is based upon low molecular weight electrophilic agents, named INACTINE™ compounds, which selectively and irreversibly inactivate nucleic acid replication.

The INACTINE compounds have the unique feature of being activated to a chemically reactive state after ionic binding to nucleic acids. This chemistry, designated as nucleic acid binding activation (NABA), provides a biochemical basis for selective toxicity to pathogens while sparing proteins, red cells and platelets. The small size of INACTINE molecules allows them to penetrate and inactivate viruses previously resistant to virucidal processes. The INACTINE technology inactivates a broad spectrum of pathogens including enveloped and non-enveloped viruses.

Recently, Pentose Pharmaceuticals for VITEX and Cangene Corporation, a Canadian biopharmaceutical company, signed a license agreement to incorporate INACTINE viral inactivation technology in Cangene’s plasma-derived therapeutic proteins. Separately, VITEX is evaluating INACTINE as a second viral inactivation method for PLAS+SD, VITEX’s approved solvent detergent pooled plasma for transfusion, marketed by the American Red Cross.

**Applying INACTINE to manufacturing processes**

To date, INACTINE has been shown to inactivate a broad panel of twenty enveloped and non-enveloped viruses. In particular, INACTINE has proven effective against several types of parvovirus, non-enveloped viruses resistant to existing inactivation methods. These viruses serve as models for human parvovirus B19, a major target for inactivation by manufacturers of plasma derivatives and a virus of concern to regulatory authorities.

For the manufacture of plasma proteins by fractionation or recombinant methods, the INACTINE technology may be applied as an upstream or downstream step in the process. For example, INACTINE may be applied at the plasma pooling stage or concurrent with the solvent detergent step. Alternatively, INACTINE may be applied downstream during the purification of individual fractions of proteins. The VITEX technical team works with licensees of its technology to identify the optimal strategy and conditions for implementing the INACTINE technology according to the production process employed.

The utilization of the INACTINE technology in a manufacturing process involves the following steps: addition of INACTINE; an incubation period; and neutralization and/or physical removal of residual compound. During the incubation period, there are three principal chemical reactions that result in modification of nucleic acid by INACTINE - 1) ionic binding to RNA or DNA, 2) nucleic acid binding activation of the compound to a reactive species, 3) covalent modification of the nucleic acid (see Figure 1). In an

![Figure 1. INACTINE structure and mode of action.](image-url)
unbound state, INACTINE compounds have low reactivity with biomolecules. However, the spatial distribution of ionic charges on the molecule allow for binding to nucleic acid phosphate groups of either single or double stranded RNA or DNA. Following the initial binding step, the INACTINE molecule is activated to a reactive form through rearrangement of its internal electronic structure. It then forms a covalent bond with the strong nucleophilic residues present on all nucleic acids. With this chemical modification, the nucleic acid of the virus cannot replicate and the virion is rendered non-infectious.

The nucleic acid binding activation mechanism is unique to INACTINE and provides a biochemical basis for selective inactivation of pathogens while sparing proteins. For example, there is greater than 85% recovery of labile proteins such as Factors V, VII and VIII from plasma under conditions that achieve a 6 log reduction of porcine parvovirus. Similarly, IgG antigen binding to rubella, measles antigen binding, and complement activation capability (CH50 activity) are all preserved. INACTINE treatment of IgG did not cause formation of dimers or changes in electrophoretic patterns with either SDS or isoelectric focusing analysis.

Once the treatment is complete, residual INACTINE compound may be chemically neutralized and/or physically removed from the manufactured product. Since INACTINE is a positively charged small molecule, it can be removed by ion exchange chromatography, size exclusion chromatography or by diafiltration. In addition, strong nucleophiles such as sodium thiosulphate, can be used to neutralize residual INACTINE.

To demonstrate conclusively the absence of additional reagents in the treated product, VITEX has developed an exquisitely sensitive analytical method to assay residual INACTINE and neutralized compound. This assay uses a modified HPLC procedure to allow the detection of the INACTINE in the picogram sensitivity range.

**Summary**

INACTINE represents a novel technology that will advance the viral safety of blood, blood derived and biopharmaceutical products. The key features of the technology are its unrivaled spectrum of virucidal action against enveloped and non-enveloped viruses combined with a selective mechanism of action that spares therapeutic elements of blood transfusion products.

For further information about this technology, contact VITEX, located in Cambridge, Massachusetts, telephone (617) 864-4800.
Current perspectives on recombinant plasma products - a regulator’s view

Summary of the speech by Professor Rainer Seitz, Paul-Ehrlich-Institut, Langen, Germany

The driving forces that lead to the introduction of recombinant coagulation factors were: the transmission of AIDS in the early 1980s and the long-known transmission of hepatitis, the limited availability of source plasma for fractionation, and a desire to obtain modified molecules that could be “tailored” for special purposes.

One of the benefits of recombinant products is that they can be assumed to be free of the typical blood-borne pathogens, provided they are not introduced by plasma-derived excipients. However, production of recombinant products has to meet special requirements, which necessitates special controls, for example, genetic stability of the product, stability of expression and post-translational modifications, as well as efficient and reliable removal of host cell materials and other contaminants during purification. The production and quality control of DNA technology products are covered in Europe by the following regulatory documents:
- Note for guidance on production and quality control of medicinal products derived by recombinant technology, III/3477/92, rev. 1994.
- Note for guidance on plasma-derived medicinal products, CPMP/BWP/269/95, rev. 2. (Contains aspects, which are also relevant for recombinant products.

Differences

Although recombinant factors possess essentially the same amino acid composition, they may still have a protein structure that differs from the natural product. This may be the cause of differences in pharmacokinetic behaviour compared with the plasma products, as was found with recombinant Factor IX. During production of recombinant products it is therefore essential to:
- Ensure that the structure is as similar as possible to the natural substance by controlling structure and conformation, post-translational changes such as glycosylation, phosphorylation and Gla-residues.
- Avoid, during expression and purification, the alteration of biological function, and activation and enhanced antigenicity because these will lead to neo-antigens and host cell impurities.

A major concern with plasma products used for haemophilia treatment is the development of inhibitors, i.e. neutralizing antibodies against the replaced factor. As a result, the impact of any product on the patient’s immune system has to be carefully assessed. It has been found that the following factors influence the manifestation of inhibitors:
- Severity of haemophilia.
- Age (usually <10 years).
- Gene defect (higher incidence with inversions, nonsense mutations, large deletions).
- Exposure days (typically 9-16, in most cases <50).
- Type of product (plasma or recombinant factor, modification of virus inactivation steps).

Clinical studies play a key role because it is not possible to predict the immunological tolerance of coagulation factor product by any kind of laboratory testing or animal model. The current CPMP/BPWP/198/95 guideline for clinical assessment of efficacy and tolerability of plasma concentrates will be revised and will also address recombinant products.

Products

Established products are rFVIII, rFIX, and activated FVII (rFVIIa), which is used for inhibitor patients as it efficiently controls bleeding. It has moderate thrombogenicity and does not contribute to induction of immune tolerance. Particulars of the rFVIIa product can be found in the European Public Assessment Report (EPAR) CPMP 729/95. Upcoming products include human inhibitors, such as antithrombin III (AT III), or activated Protein C.

Future

The extent of acceptance and utilization of recombinant and synthetic proteins in the future will depend on their overall safety compared with plasma products, on their efficacy in competition with established conventional therapy strategies, and in the overall cost-benefit ratio for these products.
During this conference, we have heard much about new technologies and new applications for many of the products we would regard as being the mainstay of this industry. This level of activity in the areas of albumin, immunoglobulins, coagulation factors and more recently fibrin sealants testifies to the underlying strength of the industry and the increasing demand for these products in an expanding variety of therapeutic applications. This increasing demand has required the industry to respond to questions of safety and purity through the incorporation of viral inactivation steps into the manufacturing process and the exploration of alternative purification strategies, often based on chromatography in these various guises, to enhance purity.

Indeed, plasma is a complex mixture of proteins. (Figure 1) It contains glycoforms, isozymes and polymers representing perhaps several thousand different molecular species. It contains latent, zymogenic or preforms of proteins, proteins that are in their active state and proteins that have been degraded or modified and are destined to be removed from the blood stream. In our single-minded approach to the isolation of a specific product, we often regard other proteins that copurify with the product of interest as “contaminants” - they are a nuisance which need to be dealt with and removed in our quest for the highest purity product. This pragmatic view obscures the reality however - many of the “contaminants” serve a function in plasma and some of these may have therapeutic potential.

Of the several thousand molecular species in plasma, we probably know something about perhaps several hundred of these. I have listed some of these on the next few slides, somewhat arbitrarily, as either existing major products or products of narrower or less developed potential. (Figure 2)
While these are major proteins, there are many other known plasma proteins with known or probable therapeutic utility. (Figure 3)

This listing is certainly not meant to be exhaustive - the message is that there are many known proteins in plasma that perhaps have yet to be fully assessed, possibly because we do not yet fully understand their function or the disease state in which they might have utility.

There is yet a third class of plasma proteins which, based on the diversity of protein species shown in Figure 1, are likely to represent unknown, unique or novel proteins in plasma. These proteins will in all likelihood not be available in quantities sufficient to service a therapeutic need. They would however provide the basis for understanding their biological roles and potential therapeutic indications.

Opposed to this view, it is reasonable to argue that proteins in plasma have been exhaustively investigated. Certainly the superb work done in the 1960’s and 1970’s resulted in a significant expansion in our knowledge base. However, it would appear that the advent of the r-DNA technology revolution in the late 1970’s and early 1980’s caused a significant redirection of research and development focus in many of the major plasma fractionators. Indeed at that time there was a widely held view that the plasma based fractionation industry would disappear completely within a decade. This view was only further reinforced by the tragedy of the HIV transmission through blood products in the early 1980’s. This further splintered R&D objectives in companies as significant resources were directed towards enhancing viral inactivation processes. The end result of these two events was a significant decline in the investigation of trace or new proteins in plasma. The technology for analysing trace amounts of proteins continued to advance unabated however. In the 1970’s, a protein chemist required nanomoles of pure protein to determine even a limited sequence of amino acids. Today, using tandem mass spectrometry for example, sequences can be derived on femtomoles of material, an increase in sensitivity of >100,000 fold. In addition our knowledge of the molecular basis of human pathogenesis has dramatically increased and, through gene knock-out and other technologies, our available animal models are becoming more credible representations of human disease states.

These factors have rekindled plasma protein research and led to the view that plasma is a rich source of proteins with novel and new therapeutic potential.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Therapeutic indication</th>
</tr>
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<tbody>
<tr>
<td>Antithrombin III</td>
<td>Hereditary/acquired Thromboembolic disorders</td>
</tr>
<tr>
<td>C-1-esterase inhibitor</td>
<td>Hereditary angioedema</td>
</tr>
<tr>
<td>Activated Protein C</td>
<td>Congenital deficiency</td>
</tr>
<tr>
<td>Haemoglobin</td>
<td>Oxygen transport</td>
</tr>
<tr>
<td>Factor VII/IIa</td>
<td>Congenital deficiency</td>
</tr>
<tr>
<td>Factor X</td>
<td>Congenital deficiency</td>
</tr>
<tr>
<td>Factor XI</td>
<td>Congenital deficiency</td>
</tr>
<tr>
<td>Factor XIII</td>
<td>Congenital deficiency</td>
</tr>
<tr>
<td>Apo-transferrin</td>
<td>Adjunct to chemotherapy</td>
</tr>
<tr>
<td>Fibrin networks</td>
<td>Drug delivery systems</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>Burns therapy</td>
</tr>
<tr>
<td>Mannose binding protein</td>
<td>Immune deficiency</td>
</tr>
<tr>
<td>α-Acid glycoprotein</td>
<td>Anti-infectious agent</td>
</tr>
<tr>
<td>C-Reactive protein</td>
<td>Antibacterial agent</td>
</tr>
<tr>
<td>Haemopexin</td>
<td>Haemolysis treatment</td>
</tr>
</tbody>
</table>

Figure 3. Other plasma proteins.