CHAPTER 27
The purification of plasma proteins for therapeutic use

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Introduction
It has been noted that blood plasma is the largest human proteome. As it is part of the circulation system, plasma contains other tissue proteomes as subsets through its contact with all organs of the body and their diverse cellular components. In addition it contains specific plasma cells involved in the immune response and homeostasis. In one study 289 proteins were detected in plasma, while in another 3700 protein spots were identified by two-dimensional electrophoresis, and 325 distinct proteins identified. Proteins found in plasma can constitute proteins secreted by solid tissues that act in plasma; immunoglobulin (Ig); "long-distance" receptor ligands, such as hormones; "local" receptor ligands, such as cytokines and growth factors; and tissue leakage products. If there is an existing disease state, there may be aberrant secretions and foreign proteins as a result of cell damage, or the presence of a tumor or infectious organism.

The true plasma proteins are considered to be those that carry out their functions in circulation. These constitute a number of proteins predominantly produced by the liver and the immunoglobulins from the bone marrow. The hepatic derived plasma proteins encompass key proteins that constitute:
• The coagulation pathway and its regulation; these include the clotting factors (fibrinogen [factor I] and factors V, VII, VIII, IX, X, XI, XII, and XIII) and the regulatory factors or protease inhibitors (antithrombin III, heparin cofactor II, and proteins C, S, and Z);
• Components of the immune response and reaction to foreign bodies via the classic and nonclassic pathway, consisting of the complement factors and mannose binding lectin;
• Acute phase reactants such as, α1-acid glycoprotein, C-reactive protein, serum amyloid protein, serum amyloid A, and α2-HS glycoprotein; constitute part of the body’s response to injury and infections; increased production of several hundredfold can occur;
• Plasma proteinase inhibitors, which regulate the action of key proteolysis enzymes such as plasmin cathepsin G, elastase, and kallikrein and include α1-antichymotrypsin, α2-macroglobulin, inter-α-trypsin inhibitor, C1 inhibitor, and α1-antitrypsin;
• Carrier proteins, which are involved in the transport of iron, copper, hemoglobin, heme, bilirubin, and fatty acids; these include albumin transferrin, ceruloplasmin, transthryretin, retinal binding protein, haptoglobin, hemopexin, and vitamin binding protein.
• Biochemical regulators involved in immune cell regulation such as interleukins, interferons, and growth factors; for example tumor necrosis factor and transforming growth factor β.

The immunoglobulins, produced in the bone marrow exist as a number of subtypes—IgG, IgM, IgA, IgE, and IgD. IgG and IgM are in the highest concentration, and have the prime role in immune surveillance, the sequestration of foreign bodies and the initiation of subsequent immune cellular and humoral responses.

Introduction
With increased understanding of the biochemical basis of disease, researchers see plasma proteins as possible therapeutic agents in a number of conditions either as a means of supplementation in cases of deficiency, or to regulate biochemical pathways to achieve desired therapeutic outcomes. The biochemistry, clinical use, and production of these proteins have been reviewed recently. Of the many identified plasma proteins, 20 therapeutic products have been developed and are in clinical use, with approximately another five in various stages of development. These are tabulated in Table 27.1.

The production of plasma proteins for therapeutic use has led to the development of a large global industry involving the complex activities of collecting blood and plasma donations and applying bioprocessing procedures to produce products with validated efficacy and pathogen safety.

It is generally agreed that the start of plasma fractionation can be traced to the work of Cohn in his Harvard Laboratory in the 1940s, where he developed an ethanol-based precipitation process for the purification of albumin to be used as a resuscitative fluid in theatres of war.

This chapter will describe the manufacture of plasma proteins for therapeutic use and make extensive use of divergent examples to illustrate the range of approaches that can be taken. In addition, it will detail the expected specifications of the final product. This information will be of use to the reader involved in the assessment, purchase, or use of plasma products, who is often confronted with literature on products containing unfamiliar concepts and terminology relating to plasma fractionation, viral removal procedures, and product specifications. An increased understanding of the production of plasma protein therapeutics will facilitate communication with plasma products manufacturers and aid in the assessment of existing products and the exploration of possibilities for future development.

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Table 27.1 Plasma proteins with established clinical use or under active investigation

<table>
<thead>
<tr>
<th>Protein or Therapeutic Product</th>
<th>Clinical Use</th>
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<tbody>
<tr>
<td><strong>Established Clinical Use</strong></td>
<td></td>
</tr>
<tr>
<td>Albumin</td>
<td>Fluid replacement in trauma, burns, sepsis, and cirrhosis</td>
</tr>
<tr>
<td>Immunoglobulins:</td>
<td>Replacement therapy in primary and secondary immunodeficiency, immunomodulation in certain autoimmune diseases including immune thrombocytopenia purpura, Kawasaki disease, Guillain–Barré syndrome, autoimmune polyneuropathy, and myasthenia gravis</td>
</tr>
<tr>
<td>- Intravenous</td>
<td>Treatment of hepatitis A, hepatitis B, cytomegalovirus, varicella–zoster, and tetanus infections</td>
</tr>
<tr>
<td>- Hyperimmune to viral and bacterial antigens</td>
<td>Prevention of Rh(D) isoimmunization due to fetus–maternal rhesus D incompatibility, treatment of immune thrombocytopenia purpura</td>
</tr>
<tr>
<td>- Anti Rh(D) immunoglobulin</td>
<td>Replacement in hemophilia A–factor VIII deficiency; induction of immune tolerance for anti-factor VIII antibodies</td>
</tr>
<tr>
<td>Factor VIII</td>
<td>Treatment of vWF deficiency</td>
</tr>
<tr>
<td>Von Willebrand factor (vWF)</td>
<td>Replacement in hemophilia B–factor IX deficiency</td>
</tr>
<tr>
<td>Factor IX</td>
<td>Treatment of patients with inhibitory antibodies to factor VIII or factor IX</td>
</tr>
<tr>
<td>Activated prothrombin complex concentrate (aPCC)</td>
<td></td>
</tr>
<tr>
<td>Composed predominately of prothrombin and factors VIII, VII, VIIa, IX, X, Xa, and protein C. Brand name: Factor VIII Inhibitor Bypassing Activity (FEIBA®)</td>
<td></td>
</tr>
<tr>
<td>Prothrombin complex concentrate (PCC); factors II, VII, IX, and X; and regulatory proteins: protein C, S, and Z</td>
<td></td>
</tr>
<tr>
<td>Fibrinogen</td>
<td>Treatment of the rare conditions of factor VII and factor X deficiencies, reversal of the anticoagulant activity of warfarin and prevention of massive bleeding, often in combination with fibrinogen</td>
</tr>
<tr>
<td>Factor XI</td>
<td>Replacement in congenital fibrinogen deficiency and in acquired deficiency following massive bleeding</td>
</tr>
<tr>
<td>Factor XII</td>
<td>Treatment of Factor XI deficiency; incidence: 1:10^6</td>
</tr>
<tr>
<td>Factor XIII</td>
<td>Treatment of Factor VIII deficiency; incidence: 1:2 × 10^6</td>
</tr>
<tr>
<td>Factor X</td>
<td>Treatment of Factor X deficiency; incidence: 1:1 × 10^6</td>
</tr>
<tr>
<td>Fibrin glue (fibrinogen and thrombin components)</td>
<td></td>
</tr>
<tr>
<td>Alpha-proteinase inhibitor</td>
<td>Used in surgery as a sealant to achieve hemostasis, as a surgical glue and to promote wound healing</td>
</tr>
<tr>
<td>Antithrombin III</td>
<td>Treatment of alpha-proteinase inhibitor deficiency</td>
</tr>
<tr>
<td>C1-esterase inhibitor</td>
<td>Treatment of acquired and hereditary deficiency of Antithrombin III</td>
</tr>
<tr>
<td><strong>Under Investigation</strong></td>
<td>Treatment of hereditary angioedema</td>
</tr>
<tr>
<td>Plasmin</td>
<td>Dissolution of clots by direct application in peripheral arterial occlusion, stroke, deep vein thrombosis, myocardial infarction, and clotted intravascular devices</td>
</tr>
<tr>
<td>Reconstituted plasma-derived high-density lipoprotein (derived from Apolipoprotein A) Transferin</td>
<td>Atherosclerotic plaque stabilization in acute coronary syndrome through reduction in lipid content and other anti-inflammatory and antithrombotic mechanisms</td>
</tr>
<tr>
<td>Plasminogen</td>
<td>Treatment of hypotransferrinemia, enhancing erythropoiesis, and reducing anaemia in β-thalassaemia. Used in hematological stem cell transplantation to sequester free iron-binding of iron reduces free-iron-mediated ischemia/reperfusion injury in transplanted organs</td>
</tr>
<tr>
<td>Haptoglobin</td>
<td>Removal of free hemoglobin arising from hemolysis in burns, trauma, and surgical intervention</td>
</tr>
<tr>
<td>Ceruloplasmin</td>
<td>Use in Ukraine and Russia has been as an adjunct therapy in cancer therapy, emergency medicine indications, and infection; outcomes thought to be enhanced by the antioxidant and ferroxidase activity of ceruloplasmin</td>
</tr>
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</table>

**Key components of the manufacture of plasma protein therapeutics**

**Plasma for fractionation**

The manufacture of plasma protein therapeutics commences with the collection of plasma. This is a logarithically and technologically complex activity due to the need to ensure product quality and safety. The viral safety of plasma collected for fractionation is ensured by what has been referred to as a “Five Layer Safety.” These layers are donor screening, blood testing, donor referral, quarantine, and investigation. Details of these activities are provided in Chapters 44, 55 and 56.

Plasma used in the United States for manufacture is either recovered plasma—plasma derived from whole blood collection, or source plasma collected by plasmapheresis. No specific monograph exists in the United States that details the handling conditions to be applied to collected plasma. However, the pharmacopeia of the European Union (European Pharmacopeia) specifically details the freezing conditions applicable for labile and nonlabile proteins. Thus, it recommends that plasma to be used in the manufacture of labile proteins needs to be frozen within 24 hours of collection, and that the rate of freezing be such that −25 °C is attained in 12 hours of being placed in the freezing apparatus. For the recovery of nonlabile proteins the plasma must be frozen to −20 °C or below within 24 hours of collection. During storage and transportation, the temperature of the plasma can exceed −20 °C for not more than a total of 72 hours, it must not transiently exceed −15 °C on more than one occasion and at no time must the temperature exceed −5 °C.

**The Cohn process**

The procedure that continues to have a pivotal role in plasma fractionation was developed by Cohn in the 1940s. It is based on the differential precipitation of plasma proteins for therapeutic use. The conditions for the generation of specific fractions are maintained at a subzero temperature. The original process, known as the Cohn method, is presented in Figure 27.1 and shows the conditions for the generation of specific fractions. Also shown is the recovery of cryoprecipitate from freeze-thawed plasma by centrifugation, typically incorporated in the processing of plasma. In addition the source of current therapeutic proteins or those under development, listed in Table 27.1, are detailed. An example will be considered to specifically illustrate the principle of the Cohn process: If one takes Supernatant I, adding ethanol to a final concentration of 25% (v/v) and adjusting the solution to pH 6.9,
while ensuring the ionic strength and temperature remain at approximately $I = 0.09$ and $-5 \, ^\circ C$ respectively, results in the generation of the immunoglobulin-enriched Fraction II + III and Supernatant II + III containing albumin.

It is important to have an understanding of the Cohn process as the terminology associated with the process is often used in literature associated with various plasma-derived products. Each Cohn fraction is an enriched, albeit crude, source of various plasma proteins that can be further purified to generate a therapeutic product. How this is achieved will be covered in the subsequent sections. Prior to this however, a description of viral removal procedures commonly used in manufacturing processes will be undertaken. These need to be incorporated in any manufacturing process to ensure the viral safety of the product.

**Viral removal procedures**

To ensure the viral safety of plasma protein therapeutics, as has been discussed in this chapter, a safe plasma supply is required, achieved through careful donor selection and screening and testing of donations. A third important determinant is the presence of inactivation or removal procedures in the manufacturing process. This is covered extensively in Chapter 56.

The purification of plasma proteins typically results, at various process steps, in the partitioning of virus away from the protein of interest. Thus, for example in the Cohn process, virus removal is achieved with the precipitation of Fractions III, I/III, I/II/III, and Fraction IV-I/IV-4 in the manufacture of immunoglobulin and albumin, respectively. Regulatory requirements, however, require the inclusion of at least two dedicated orthogonal viral removal procedures that are effective against both enveloped and non-enveloped viruses.

There is an ongoing interest in identifying effective yet convenient means of achieving viral removal. Viral filtration is a preferred option as it can, in many cases, be added to an existing process and is usually benign with respect to its effect on product integrity. The preferred pore size for viral filters is 15 or 20 nm, thereby...
ensuring the removal of small non-enveloped viruses such as parvovirus B19 and hepatitis A.\(^{45}\) This can limit the size of the protein that can be filtered. Immunoglobulins with a molecular weight of 150,000 d, can readily be filtered.\(^{46,47}\) For larger proteins, viral filters with 35 nm pore size need to be used, although this would only be effective in removing large enveloped viruses such as hepatitis B and C and HIV. Examples of this include von Willebrand factor (vWF) which has a molecular weight of 500,000 d but exists as a series of multimers to a molecular weight of 20,000 kd.\(^{58}\) Factor VIII–vWF has been filtered through a 20 nm filter by using specific conditions to dissociate the complex prior to filtration and subsequent inducing reassembly.\(^{49}\) Viral filtration of fibrinogen, with a molecular weight of 340,000 d, through a 20 nm filter has also been achieved.\(^{50}\)

**Chromatographic procedures**

The Cohn fractionation process can generate highly purified albumin and immunoglobulin products.\(^{32,51}\) Today, albumin is still predominately purified by the Cohn procedure, and this was the case until the late 1990s for the production of immunoglobulin products. The purification of other therapeutic proteins from enriched Cohn fractions has only been able to be achieved by the application of chromatographic techniques.\(^{52}\)

Details of the purification of the major plasma-derived therapeutic proteins will be provided in this chapter. However, an appreciation and understanding of these processes requires knowledge of the principles of chromatography and the orthogonal approaches that need to be applied to obtain highly purified preparations.

Chromatographic techniques allow the purification of proteins because of their differences in molecular weight, charge, hydrophobicity, and specific affinity for ligands. This involves the use of size exclusion, ion exchange, hydrophobic interaction (HIC), and affinity chromatography, respectively.\(^{53–56}\) Chromatography is typically performed with columns packed with resin beads of about 80 µm in diameter and derivatized with particular functional groups to allow the particular chromatographic separation to be performed.

Size exclusion separation occurs as proteins percolate through the beads. Smaller proteins enter the pores of the beads, and hence their passage through the column is retarded, leading to the separation of proteins on the basis of molecular weight, with higher molecular weight proteins eluting first followed by the smaller species.

Ion exchange chromatographic resin exist in either the anion (positively charged) or cation (negatively charged) forms. Typical ion exchange ligands include diethylaminoethyl (DEAE), quaternary amino ethyl (QAE), quaternary ammonium (Q), carboxymethyl (CM), sulfopropyl (SP), and methyl sulfate (S) for anion and cation exchange chromatography, respectively. As proteins are zwitterion molecules, the net charge of the molecule can be modulated by changing the pH of the protein environment. Proteins have different pI values—the pH at which the net charge of the protein is zero.\(^{56}\) Therefore, the net charge of a protein depends on pH, and this determines the degree of interaction with an ion exchange resin. Interaction can be further modulated by changes in ionic strength. Thus, by utilizing conditions that promote differential binding to ion exchange resins, coupled with defined elution conditions—again involving specific conditions of pH and conductivity—fractionation of a mixture of proteins can be achieved.

Proteins exhibit differences in their hydrophobic profile, reflecting differences in the composition of amino acids with hydrophobic (nonpolar) or hydrophilic (polar) properties.\(^{56}\) This difference is exploited in hydrophobic interaction chromatography (HIC).

Solution conditions can be manipulated so that the more hydrophobic proteins are retained on a column while less hydrophobic proteins flow through.

Proteins exhibit unique surface epitopes and functional domains.\(^{56}\) Immobilized antibodies and specific ligands on chromatographic resins, which can specifically interact with these parts of proteins, can be the basis of affinity chromatography for the purification of proteins from complex mixtures. Immobilized monoclonal antibodies, heparin, and metal affinity ligands are used in the purification of several plasma proteins.\(^{52}\)

**Manufacturing processes for plasma protein therapeutics**

In this section, specific processes used in the recovery of all the major plasma proteins for therapeutic use will be described. The focus will be on illustrating the range of techniques that can be used in the manufacture of these products, and exposing the reader to a wide range of concepts underpinning the fractionation of plasma proteins. In addition, the expected pharmacopeial specifications of the final product, that ensure desired product safety and efficacy, will be detailed.

**Albumin**

The Cohn process remains the dominant process for the manufacture of albumin (Figure 27.1).\(^{32}\) Details on the production of albumin by the Cohn process in a modern facility and the required quality attributes expected of the product have been reviewed elsewhere.\(^{19}\)

As previously described, the Cohn process involves manipulation of ethanol concentration and pH of solutions at low ionic strength, while maintaining subzero temperatures, to achieve differential precipitation of proteins. The recovery of albumin involves the generation of Supernatant I, II+III, IV-I, IV-4, and finally the precipitation of pure albumin as Fraction V at 40% (v/v) ethanol, pH 5.8, with temperature maintained at −5°C. A variation to the Cohn process was reported by Kistler and Nitschmann in 1962.\(^{57}\) The aim was to maximize albumin yield and decrease the use of ethanol. Fraction I is generated as by the Cohn process. Precipitate A, the equivalent of Fraction II+III in the Cohn process, is produced with 19% (v/v) ethanol, pH 5.85, and allows the recovery of the albumin containing Supernatant A. This then progresses through the generation of Supernatant IV (40% v/v) ethanol, pH 5.85, −8°C) and Precipitate C (40% v/v) ethanol, pH 4.8, −8°C. Resuspension of Precipitate C and recovery of Supernatant D (10% v/v) ethanol, pH 4.6, −3°C) provide the final purified albumin. This process was initially used by the Swiss manufacturer ZLB, and continues to be used following its incorporation into CSL Behring.\(^{31}\) The albumin recovered by ethanol fractionation is highly purified, although trace amounts of residual plasma proteins—typically, haptoglobin, ceruloplasmin, α1-macroglobulin, acid–glycoprotein, hemopexin, and transferrin—can be detected.\(^{58,59}\) (CSL data on file).

An alternative chromatographic process, based on the method of Curling et al., was developed by CSL Behring (Australia) in the 1990s.\(^{58,60,61}\) The process was initially developed for the purification of albumin from Supernatant II + III but was later adapted for the processing of Supernatant I.\(^{62}\) The process is shown in Figure 27.2. The process incorporates three chromatographic steps—anion and cation exchange chromatography with DEAE and CM Sepharose, respectively, and size exclusion chromatography using Sephacryl S200 resin. This last step markedly contributes to the purity of the recovered albumin, removing residual proteins and aggregates.
Chromatographically purified albumin is purer than that derived by ethanol fractionation and also exhibits a lower aggregate content. This increased purity has been associated with a decreased rate of adverse reactions with chromatographically purified albumin.

The Cohn process for the manufacture of albumin contributes to the viral safety of the product through partitioning virus away from the product stream. With the alternative chromatographic process, similar partitioning is achieved at the chromatographic steps. In addition, a specific viral inactivation step is included in the manufacturing process—pasteurization at 60 °C for 10 hours. This has been part of the production of albumin from the very beginning and undoubtedly accounts for the fact that there has never been a viral transmission from an albumin product. The pasteurization of albumin was possible due to the finding that N-acetyl tryptophanate and sodium octanoate (caprylate), or sodium octanoate alone, stabilized the molecule during heating.60–71

The European Pharmacopoeia (EP) and US regulations (CFR21) stipulate that pasteurization be performed in the final container following dispensing in order to completely remove the risk of recontamination. Reflecting advances in bioprocessing execution and control, CSL Behring (Australia), in addition to developing a novel chromatographic manufacturing process, obtained approval from the Australian regulatory authority to implement bulk pasteurization of its albumin product prior to dispensing. This product (Albumex®) is registered and used in Australia, New Zealand, Hong Kong, Singapore, Taiwan, Malaysia, and Indonesia (CSL data on file). Albumex® has an additional viral inactivation step consisting of incubation at low pH in the presence of caprylic acid at 30 °C for 10 hours. This step was introduced to add further viral safety to the product and reflected the regulatory expectations that plasma protein products have at least two dedicated viral inactivation or removal steps.

The specifications of the product are governed by EP and US Pharmacopoeia and National Formulary (USP) or CFR pharmacopeial requirements, which provide limits for physical properties, biological safety, purity, excipients, and contaminants. Purified albumin solutions are supplied by various manufacturers as 4.5, 5, 20, and 25% (w/v) solutions and typically exhibit a shelf-life of up to five years at room temperature (25–30°C). It is a requirement that solution must be clear and can be almost colorless, yellow, amber, or green (EP). The variation in color reflects differences in the presence of residual heme as well as colored protein impurities such as haptoglobin, ceruloplasmin, transferrin, and hemopexin. Chromatographically purified albumin is typically a greenish color, reflecting its higher purity. It contains only a small amount or absence of colored proteins and heme, but increased levels of albumin bound bilirubin and the green oxidized derivative—biliverdin. The product should not change in appearance with heating at 57 °C for 50 hours (CFR21).

The product should have a pH (at 20 °C) of 6.7–7.3 (EP) or 6.4–7.4 (USP). Osmolality should be equivalent to that of plasma (USP). With respect to excipients, sodium should not exceed 160 mmol/L (EP) or 130–160 mmol/L (USP). Sodium caprylate (sodium octanoate) should be 0.16 mmol/g albumin if it is the single stabilizer (CFR21). In preparation where dual stabilizers, sodium caprylate and N-acetyl tryptophanate, are used, the concentration of each should be 0.08 mmol/g albumin (CFR21).

Albumin purity should be at least 95% (EP) or 96% (USP), with aggregate content not more than 10% as determined by the area of the chromatogram determined by adsorption at 280 nm. Heme levels as determined by adsorption at 403 nm should not exceed 0.15 AU (EP). Chromatographically purified albumin contains no detectable heme-related absorbance.

Prekallikrein activator (PKA) activity should not be more than 35 IU/mL (EP). This is an important specification as PKA (also known as factor XIIa or Hageman factor fragment), if present, can lead to a clinical hypotensive reaction.

Contaminant levels, of which potassium and aluminum are specified, should be 0.05 mmol/g albumin (EP) or 2 meq/L (USP) and 200 μg/L (EP), respectively. The limit for aluminum was introduced into the British Pharmacopoeia in 1993 to ensure albumin safety for use in renal dialysis and with premature babies. Another concern was the accumulation of aluminum with the treatment of burn patients. The source of aluminum is from the glass containers used for the product. Leaching of aluminum is abetted by even very low concentrations of citrate (<0.1 mmol/L), which originates from the anticoagulant used during plasma collection. Through optimization of dialfiltration processes used in manufacturing, to minimize citrate content, current albumin products meet the required aluminum pharmacopeial limit.

The solution should be sterile, nonpyrogenic, and with endotoxin levels not more than 0.5 EU/mL (EP/USP). The pharmacopoeias also specify that the final product be incubated for a period prior to final inspection to provide additional assurance of product sterility. CFR21 requires incubation for at least 14 days at 20–35°C, and the EP states not less than 14 days at 30–32 °C, or not less than four weeks at 20–25 °C.

### Immunoglobulins

The Cohn process also served as the starting point for the purification of immunoglobulins. Oncley developed a process for the recovery of purified immunoglobulins from Cohn Fraction II + III. In the process, the fraction is resuspended and reprecipitated with 20% v/v ethanol, at pH 7.6 (modifications exist with pH 6.7), and temperature at −5 °C. The recovered precipitate is then re-suspended, and the solution adjusted to 17% v/v ethanol, at pH 5.2, with temperature maintained at −6 °C. This results in precipitation of IgM and other impurities (Fraction III), whereas IgG remains in
the supernatant (Supernatant III). IgG is then recovered as Fraction II by precipitation with 25% v/v ethanol, pH 7.4, with the temperature at \(-5^\circ C\).

A modification of the Oncley process was developed by Kistler and Nitschmann in 1962. The aim was to increase the yield of IgG and reduce ethanol use. In this process, Precipitate A (corresponding to Cohn Fraction I + II + III) is obtained at 19% v/v ethanol, pH 8.5, temperature \(-5^\circ C\). Following resuspension of the precipitate in water, the solution is adjusted to pH 5.1 and ethanol is added to 12% v/v while maintaining the temperature at \(5^\circ C\). This results in the precipitation of impurities, including IgM (Precipitate B), with IgG remaining in the supernatant (Supernatant b) (equivalent of Cohn Supernatant III). The IgG is recovered by precipitation at 25% v/v ethanol, pH 7.0, at \(-7^\circ C\). This precipitate G corresponds to Cohn Fraction II. These processes, with various modifications, for many years, have accounted for the bulk of commercially produced immunoglobulins. The Kistler–Nitschmann process was particularly identified with the ZLB (later CSL Behring) facility in Bern, where the process was used for the production of Sandoglobulin.

The development of new immunoglobulin manufacturing processes from the mid-1990s has reflected the desire of manufacturers to improve product characteristics and safety. This has led to the increase in final formulation protein concentration in order to increase the convenience of use, the introduction of viral inactivation steps to improve safety; and the introduction of procedures to improve yield and manufacturing efficiency. A number of companies have developed hybrid processes where the crude IgG precipitate generated by ethanol precipitation (Fraction II + III or Precipitate A) is subjected to further chromatographic processing to recover the purified immunoglobulin. CSL Behring (Australia) developed a unique completely chromatographic procedure, and in 2000, its use commenced for the production of immunoglobulin. A key feature of this process was the increased yield in IgG that was achieved when compared to the Cohn-based process.

In the following, a brief description is presented of the manufacturing processes of all the major immunoglobulin products currently produced and the expected pharmacopeial specifications of immunoglobulin products. This area has also been reviewed extensively elsewhere. A number of products are purified essentially by the complete application of the Oncley ethanol fractionation process. In the manufacture of Octagam (Octapharma), purified IgG is recovered as Fraction II (Figure 27.3). In addition to the viral partitioning that occurs during the fractionation process, there is solvent–detergent (S/D) treatment and low-pH incubation, as described above, for viral inactivation. The oil/solid phase extraction step is a means of removing the S/D used during the viral inactivation procedure.

In the BIVIGAM (Biotest, AG) process, pure IgG as Supernatant III is produced by the Oncley process. This is then subjected to the first viral inactivation procedure involving S/D treatment. It is then further purified by Q anion exchange chromatography before undergoing viral filtration through a 35 nm pore filter (Figure 27.4). The specific viral inactivation procedures in this process target inactivation and removal of enveloped viruses (HIV, hepatitis B, and hepatitis C). Removal of non-enveloped viruses (parvovirus B19 and hepatitis A) relies on partitioning into the discarded Fraction III during the recovery of Supernatant III.
KIOVIG® or Gammagard® produced by Baxalta (European and US trade names, respectively) is also produced by the Oncley process resulting in the generation of purified IgG as Fraction II+III (Figure 27.5). The purified immunoglobulin is then subjected to three viral inactivation or removal procedures—S/D treatment, viral filtration with a 35 nm filter, and low-pH formulation and incubation of the dispensed product. The cation exchange chromatography step is used to bind the immunoglobulin and thereby allow the removal of the S/D used in the viral inactivation procedure. Passage through the anion exchange column retains impurities and results in further purification of the immunoglobulins.

The manufacture of Gamunex® (Grifols) is one of a number of hybrid manufacturing processes that involves the generation of purified IgG as Fraction II+III (Figure 27.6). Cohn Fraction II + III is suspended and caprylic acid added to precipitate impurities. The caprylic acid concentration is adjusted in the recovered filtrate and incubated at 25 °C for one hour. This constitutes the first viral inactivation procedure in the manufacturing process. The generation of pure IgG is achieved by sequential passage through a strong (Q) and a weak (DEAE) anion exchange chromatographic columns. Following buffer exchange, concentration, and formulation, where pH is adjusted approximately 4.25, the product is dispensed and then incubated at 28 °C for 21 days. This constitutes the second viral inactivation step. The viral inactivation procedures of Gamunex® are effective for enveloped viruses (HIV, hepatitis B, and hepatitis C) but are not recognized for use against non-enveloped viruses (hepatitis A and parvovirus B19). The virus clearance for non-enveloped virus in the Gamunex® process is accounted for by caprylic acid precipitation, depth filtration following the caprylate incubation, and partitioning during anion exchange chromatography.

In the manufacture of Flebogamma DIF®, Cohn Fraction II + III is resuspended, partially purified by precipitation of impurities by the addition of polyethylene glycol (PEG), and then further purified by anion exchange chromatography under conditions whereby impurities are retained. The purified immunoglobulin recovery is subjected to three validated viral removal or inactivation procedures: pasteurization, S/D treatment, and viral filtration through 20 nm filters. The PEG precipitation of the resuspended Cohn Fraction II + III is also associated with significant viral clearance.

There is a second PEG precipitation after the low-pH treatment, pasteurization, and S/D treatment, which serves to remove immunoglobulin aggregates and facilities passage of the solution in the subsequent viral filtration step (Figure 27.7).

The purification of Clair Y® (LFB) involves the use of Cohn Fraction I + II + III. Following resolubilization, impurities are removed by caprylic acid precipitation. The filtrate is ultrafiltered to remove caprylic acid and the retentate is subjected to S/D treatment. The immunoglobulin is then captured on an anion exchange column allowing the solvent and detergent of the viral inactivation procedure to be removed. The eluted immunoglobulin
is then passed through an affinity resin to reduce the anti-A and anti-B isoagglutinin titer. The immunoglobulin then undergoes viral filtration, the second viral removal step, prior to formulation and filling (Figure 27.8).

The manufacture of Privigen® (CSL Behring) is also a hybrid manufacturing process that involves the generation of a crude IgG fraction by ethanol precipitation followed by further purification by chromatography. Kistler–Nitschmann precipitate A or Cohn Fraction II + III is resuspended, and caprylic acid addition is used to precipitate impurities. The recovered partially purified immunoglobulin solution is subjected to low-pH incubation in the presence of a low concentration of detergent as part of the first viral inactivation procedure incorporated in the process. Final purification of the immunoglobulins is achieved by passage through a Q anion exchange chromatographic column under conditions where impurities (predominantly IgM and IgA) are bound and IgG flows through. This in turn is passed over an affinity column to decrease the anti-A and anti-B hemagglutinin titers. The recovered immunoglobulin solution is then subjected to viral filtration the second viral removal procedure in the process, prior to formulation and dispensing (Figure 27.9).

The final manufacturing process to be described is a completely chromatographic process used by CSL Behring (Australia) to manufacture Intragam P® (Figure 27.10). As has been mentioned in this chapter, a key feature of this process is the achieved increased recovery of immunoglobulins. Supernatant I, which is
The process includes pasteurization as the first viral inactivation procedure. Following formulation at a low pH (pH 4.25) and filling, the dispensed product is incubated at 27°C for 14 days as the second viral inactivation procedure of the process. Gamimune N® (Miles Cutter/Bayer) in 1992 was the first immunoglobulin for intravenous use to be available as a 10% (w/v) formulation. There has been a consistent trend over the last 20 years of moving from a final protein concentration of 5 or 6% (w/v) to 10% (w/v). The increased protein concentration reduces the volume that needs to be infused and hence, is more convenient for the patient. Today, all the major immunoglobulin products on the market that have been described here are available at the 10% w/v formulation. CSL Behring has developed a 20% w/v formulation immunoglobulin product called Hizentra® intended for subcutaneous administration.

A key challenge in the development of an immunoglobulin preparation suitable for intravenous use was identifying a means of preventing the development of anti-complementary activity in the product arising from the formation of aggregates. These led to spontaneous activation of the complement system and caused severe adverse reactions. Early success in inactivating anti-complementary aggregates was achieved in 1962 by Barandum and Islíker through mild proteolysis with pepsin at pH 4.0. Improvement in processing, such as the use of ultrafiltration and diafiltration at pH 4.0 for the removal of ethanol from Supernatant III and for the concentration of IgG, instead of precipitation and lyophilization, minimized aggregate formation and generated a clinically tolerated product. It was then shown that formulation of the immunoglobulin at approximately pH 4.25 generated a liquid stable product with low anti-complementary. The stability of immunoglobulin at low pH is due to the fact that this is below their isoelectric point, and results in non-interacting positively charged molecules. Consequently, many immunoglobulin products are now formulated at less than pH 5.0. This is the case for many of the products described above—Privigen®, Hizentra®, KOIVIG® (Gammagard®), BIVIGAM®, Intragam P®, and Gammunex®. Formulation pH of Flebogamma DIF® and Octogam® is still much lower than neutral at pH 5.6-6.0, respectively.

Excipients are used in the formulation of immunoglobulins. These serve to stabilize the immunoglobulin molecules in solution by minimizing protein–protein interactions to ensure the clinically acceptable purity of the product. Most immunoglobulin products in fact can be stored at room temperature for up to 36 months. Currently used excipients comprising of amino acids, sugars and sugar alcohols have a long history of use and are known to be well tolerated. Glycine is most commonly used, but as illustrated by the products described above, proline, maltose, sorbitol, and mannitol are also used. Low concentrations of sodium chloride and polysorbate detergent may also be added to adjust tonicity and enhance stability.

As immunoglobulins for intravenous use are manufactured by varying processes, the properties of the different products produced have been examined. In addition, consideration has been given to whether formulation differences could affect clinical tolerability. Although differences have been noted, there is no evidence that they significantly correlate with differences in product efficacy and tolerability. As registered products, these immunoglobulin products meet the standards as prescribed by relevant regulatory authorities.

The European Pharmacopoeia has a comprehensive monograph on immunoglobulins for intravenous use. The product is expected to be sterile and free of pyrogens and endotoxins. A liquid formulation is expected to be clear or slightly opalescent, colorless, or pale yellow. The allowable pH is 4.0–7.4. Osmolality must be greater than 240 mOsmol/kg. The minimum allowable protein concentration of a formulation should not be less than 30 g/L (3% w/v). As modern immunoglobulin products are typically formulated at 10% (w/v), this limit is not usually relevant. Purity is expected to be greater than 95%—with the currently used manufacturing processes, this is readily achieved. Aggregate content must not exceed 3% of total protein. This is an important quality attribute as aggregates typically result in complement activation-mediated adverse reactions. Any propensity for this is monitored through the measurement of anti-complementary activity, with the specification set at <1 CH50/mg IgG. Specifically, this means that an aliquot of immunoglobulin solution will not sequester more than 50% of a given amount of complement.

The allowable maximum limit of prekallikrein activator (PKA) (factor XIIa), which can mediate the formation of the vasoactive peptide bradykinin from kinnogen and lead to hypotensive reactions, is set at 35 IU/mL. Hemagglutinins, anti-A and anti-B antibodies to red cell antigens, can result in hemolytic reactions with the infusion of immunoglobulins. Therefore, the EP stipulates a maximum allowable titer of 1:64. Given that high-volume administration of immunoglobulins is required in some indications, specifically those associated with achieving immune modulation, manufacturers are introducing specified hemagglutinin removal steps in...
manufacturing their process to lower the titer in their products. Therefore, the titer of some products will be considerably lower than this limit.

The EP does not define a maximum level of IgA allowed in immunoglobulin products but the product must comply with the level stated on the label. The range for the products described here is $5–200\ \mu\text{g/mL}$. Knowledge of the IgA content of an immunoglobulin product is important for a clinician when confronted with an IgA deficient patient who could exhibit an anaphylactic reaction if infused with a higher IgA concentration immunoglobulin product.

Although not prescribed in the EP, immunoglobulin products are expected to exhibit a subclass distribution comparable to that normally found in plasma. Partial depletion of IgG3 and IgG4 is, however, encountered in a number of manufacturing processes. There is no evidence that this affects the efficacy of the product. The EP requires that a developed immunoglobulin purification process maintain the integrity of the Fc portion of the immunoglobulin molecule—key to its immunomodulatory role through interaction with the Fc receptor and its effector role in complement activation. Monitoring of Fc function is not a product release requirement but is part of process validation. An immunoglobulin product should exhibit an Fc function of $>60\%$ of that of an EP standard. However, current products typically have values of $100\%$.

The EP also stipulates that antibody titer to hepatitis B be at least $0.5\text{IU/g IgG}$. There is no limit for antibodies to hepatitis A, but regulatory authorities expect levels to be above $10\text{IU/mL}$. This may be increasingly difficult to maintain due to the consistent decline in population titers from at least 2003 due to increased hygiene and the availability of an effective hepatitis A vaccine. Product specifications for the United States also include minimum antibody levels for measles, diphtheria, and polio. With respect to measles, there are interesting trends suggesting that as disease prevalence is decreasing as a result of increased vaccination, antibody titer in plasma and hence immunoglobulin products is decreasing.

In 2010, increased incidences of thromboembolic events were reported in patients using Octogam. Analysis showed this was due to increased levels of factor Xa caused by a process change. The regulatory agencies requested that manufacturers confirm that their processes have the capability of removing thrombogenic activity and generate a product low in procoagulant activity. Procoagulants have been shown to be partitioned away from the product during purification and inactivated by pasteurization and exposure to low pH. The most commonly used methods are non-activated partial thromboplastin time (NaPTT) and the thrombin generation assay (TGA). The latter is typically calibrated relative to factor Xa concentration. NaPTT values with clotting time $>150$ seconds are considered acceptable. With respect to TGA-derived results, existing products typically have very low to nondetectable levels of factor Xa.

**Factor XIII**

The development of manufacturing processes for factor XIII has been extensively reviewed. The focus here will be on describing the production of a major commercial factor XIII product, Fibrogammin P (in Europe) and Corifact (in the United States), as undertaken by CSL Behring. The process utilizes Cohn Fraction I precipitate derived from cryoprecipitate-depleted plasma. Following resolubilization and removal of fibrinogen by treatment with aluminum hydroxide and Vitacel (a cellulose fiber), the clarified solution is subjected to DEAE anion exchange chromatography.

The purified factor XIII is then subjected to two viral inactivation and removal procedures—pasteurization ($60\ ^\circ\\text{C}$ for 10 hours) and viral filtration with $20\text{nm}$ filters. These dedicated viral removal procedures, together with viral partitioning achieved during the chromatography procedure, result in excellent viral clearance through the manufacturing process. Following formulation to the target potency and with the addition of albumin, glucose, and sodium chloride, the solution is sterile filtered, dispensed, and lyophilized. There is no monograph for factor XIII product, but these products have been extensively characterized with respect to purity, function, and factor XIII integrity. This includes factor XIII activity, specific activity, factor XIII subunit A content, fibrinogen clotting time, PKA, and activated factor XIII.

**Figure 27.11** Manufacturing process for factor X (BPL).
metal affinity chromatography with a copper chelate resin. Viral removal of enveloped and non-enveloped viruses is achieved by S/D treatment and viral filtration with 15 nm filters. In addition, the lyophilized product is subject to heat treatment at 80 °C for 72 hours. The product has been shown to comply with required toxicity, thrombogenicity, and immunogenicity requirements. In addition, in vitro tests confirm the product is potent, highly purified, and free of potentially hazardous residues. The testing includes determination of factor X potency, specific activity, the presence of factor II, factor IX, proteins C and S, and NaPTT.

Activated prothrombin complex concentrate (aPCC)
aPCC, known as factor VIII inhibitor bypassing activity (FEIBA®), is manufactured by Baxalta. Details on the development, production, mechanism of action, and clinical use of the product have recently been reviewed. The manufacturing process is shown in Figure 27.12. It involves purification of prothrombin complex factors (II, VII, IX, and X) from cryoprecipitate-depleted plasma by DEAE anion exchange chromatography. The recovered material undergoes viral filtration with a 35 nm filter. The factors are then contact activated, generating activated Factor VII and some activated factor X. These activated factors, together with prothrombin, are thought to be important for the clinical efficacy of the product. The product undergoes a second viral inactivation treatment involving vapor heat treatment at 60 °C and 80 °C for at least 8.5 hours and 1 hour, respectively, on the lyophilized product. This step is effective against both enveloped and non-enveloped viruses. The product is then formulated with sodium citrate and sodium chloride, and dispensed in 500, 1000, and 2500 arbitrary FEIBA units. One unit of activity is defined as that amount of product that shortens the activated partial thromboplastin time (aPTT) of a high-titer factor VIII inhibitor reference plasma to 50% of the blank value. The final product is a lyophilized preparation.

Alpha2-proteinase inhibitor
The manufacturing processes for the four major alpha2-proteinase inhibitor (API) products on the market, Aralast NP® (Baxalta), Zemaira® (CSL Behring), Prolastin C® (Grifols), and Glassia® (Kamada), are presented in Figure 27.13. A comprehensive examination of these manufacturing processes is available elsewhere.

Cohn Fraction IV-I is the source material. The Aralast NP® process utilizes a unique step involving, an extended thaw of the paste and dissolution in pH 6.0 buffer that allows the recovery of an API-enriched precipitate. The generated initial crude API solutions undergo an initial “activation hold” step at approximately pH 9 and at an elevated temperature of approximately 45 °C. This results in increased recovery of functional protein, which could be related to refolding of denatured protein. The commencement of the purification process typically involves polyethylene glycol (PEG) precipitation of impurities, destabilization of unwanted proteins by reduction with dithiothreitol (DTT), and adsorption with fumed silica (Aerosil®). This treatment takes advantage of the fact that there are no disulfide bonds in the API molecule. All processes utilize anion exchange chromatography for further purification. Further polishing is achieved by adsorption with bentonite (aluminum phyllosilicate clay), hydrophobic interaction chromatography, or cation exchange chromatography. The Aralot NP®, Glassia®, and Prolastin-C® processes all incorporate S/D treatment as the first viral inactivation procedure. Pasteurization is used in the Zemaîra® process. All processes include viral filtration as the second viral removal step, and this is effective for both enveloped and non-enveloped viruses. Aralast NP®, Zemaira®, and Prolastin C® are presented as lyophilized final products, whereas Glassia® is a liquid product.

The EP prescribes certain product qualities for API preparations. The specific activity should not be less than 0.35 mg of active API per mg of total protein. The ratio of API activity to antigen should not be less than 0.7. With respect to appearance, the liquid preparation should be clear or slightly opalescent, colorless, or pale yellow, pale green, or pale brown; the freeze-dried preparation should be a powder or solid friable mass, hygroscopic, and white, pale yellow, or pale brown.

It is required that the pH be in the range of 6.5–7.8, that the preparation be completely soluble, and that osmolality be greater than 240 mOsmol/kg. In addition, the water content of the freeze-dried preparation must be within the limits approved for the product by the competent authority. The product must be sterile and free of pyrogens and bacterial endotoxins.

Factor XI
The manufacture of factor XI is undertaken by LFB Biotechnologies (France). Cryoprecipitate-depleted plasma (cryosupernatant) is passed through a negatively charged filter to capture the factor XI. It is then eluted with 1 M NaCl containing antithrombin III (ATIII), which inhibits any proteolytic activity that could degrade the product. Following buffer exchange by ultrafiltration, the solution is subjected to viral inactivation treatment with S/D. The solution is passed through a sulfate(S) cation exchange chromatographic resin to capture the protein and allow the washing away of the virus-inactivating chemicals. The factor XI is recovered with eluate buffer containing lysine and arginine by increasing the salt content and pH to 170 mM and 7.5, respectively. Heparin and ATIII were added to the eluate, and then it was sterile.
filtered, dispensed, and lyophilized. The product is highly purified, containing only trace amounts of fibrinogen, albumin, C1-esterase inhibitor, fibronectin, alpha2-macroglobulin, and IgG. The levels of other coagulation factors, kinin system components, and proteases are also low or nondetectable.\textsuperscript{139}

The EP required that the freeze-dried final product be white or almost white powder or friable solid. The water content must be within the limits approved by the competent authority. It should completely dissolve within 10 minutes. The potency must be within 80–120% of that stated on the label. The reconstituted product should be of pH 6.8–7.4 and have a minimum osmolality of 240 mOsmol/kg. Heparin and ATIII added as stabilizers must comply with the level stated on the label. Ensuring the absence of activated coagulation factors, the NaPTT coagulation time should not be less than 150 seconds. There should not be any significant presence of anti-A and anti-B hemagglutinins, with no agglutination occurring at a 1:64 dilution of the product. The product must be sterile and free of bacterial endotoxins.\textsuperscript{140}
A recent review comprehensively deals with the biology, purification, and clinical use of C1-esterase inhibitor. This section will focus on describing the manufacturing processes of two of the major C1-esterase inhibitor products on the market: Berinert® (CSL Behring), and Cinryze® (US) or Ceton® (Europe) (Sanguine). As can be seen in Figure 27.14, the processes are quite distinct in the use of chromatographic processes. In the production of Berinert®, cryoprecipitate-depleted plasma is initiated; passed through a DEAE anion exchange column, under conditions in which the C1-esterase inhibitor flows through; and subsequently captured on a QAE anion exchange resin. Further purification is achieved by ammonium sulfate precipitation steps and hydrophobic interaction chromatography (HIC). In the Cinryze® process, the C1-esterase inhibitor is captured from the starting material by anion exchange chromatography, and further purification is achieved by PEG precipitation and two procedures involving cation exchange chromatography. In both processes, viral removal involves pasteurization and virus filtration requiring two filters in series. The recovered virally filtered product is formulated, dispensed, and lyophilized. In the case of Berinert®, excipients used in its formulation are glycine, sodium chloride, and sodium citrate.

There are no pharmacopoeial requirements for C1-esterase inhibitor concentrates. However, as with other lyophilized therapeutic protein products, it must have an acceptable appearance in the lyophilized state and comply with registered residual moisture content. The product must readily dissolve and present as a colorless and clear solution with clinically acceptable osmolality and insoluble particle count. It must be sterile and free of bacterial endotoxins. Manufacturers determine and report the potency of the product. The integrity of the product is determined by monomer content, which is a quality control release specification. Thus, for example, Berinert® must have a monomer content of >89% to meet release specifications (CSL data on file). Recently, a biochemical and purity comparison of four commercially available C1-esterase inhibitor products (Berinert®, Cinryze®, Cetor®, and Ruconest® [transgenic product]) was published consisting of characterization of molecular weight, determination of specific activity, and quantitation and identification of residual proteins. It was the hope of the authors that the work would contribute to the establishment of regulatory requirements for determining purity and setting allowable threshold levels.

Antithrombin III
As examples of manufacturing processes for Antithrombin III (ATIII), the processes for Kybernin P®, Thrombotrol® (CSL Behring [Australia]), and Thrombate III® (Grifols) are...
described and shown in Figure 27.15. Further information on the biology, purification, and clinical indications for ATIII is available in a recent review.

From either Supernatant I, cryoprecipitate-depleted plasma, or a Fraction IV-I suspension, ATIII is captured by heparin affinity chromatography. The Kybernin P® process then uses sequential ammonium sulfate steps to remove impurities and to concentrate the ATIII by precipitation. All processes incorporate pasteurization as a viral inactivation step. In the Kybernin P® process, a subsequent ammonium precipitation of ATIII allows the removal of pasteurization stabilizers and the production of a concentrated solution, which is formulated, dispensed, and lyophilized. In the Thrombotrol® and Thrombate III® processes, further purification is achieved by size exclusion and heparin affinity chromatography, respectively. Both processes incorporate viral filtration. The recovered filtrate is then concentrated and diafiltered prior to formulation, dispensing, and lyophilization. Thrombotrol® and Thrombate III® processes incorporate two dedicated viral inactivation and removal steps that are effective for both enveloped and non-enveloped viruses. The Kybernin P® process has only one dedicated viral inactivation step—pasteurization, which is effective against both enveloped and non-enveloped viruses. In addition, viral clearance is achieved by precipitation at the first ammonium sulfate precipitation step.

The EP prescribes a number of product specifications. At least a 60% fraction of the product must bind heparin. The specific activity of the product should not be less than 3 IU ATIII per mg of protein (excluding albumin excipient). The lyophilized product should be a white or almost white, hygroscopic, friable solid or powder. The water content must be within limits approved by the competent authority. The product should completely dissolve within 10 minutes with gentle swirling, giving a clear or slightly turbid, colorless or almost colorless solution. The limits for pH and osmolality are 6.0–7.5 and >240 mOsmol/kg, respectively. The maximum allowed heparin content is 0.1 IU per IU ATIII. The product must be sterile and free of bacterial endotoxins.

**Fibrinogen**

Riastap® (US) or Haemocomplettan® (Europe) produced by CSL Behring is the leading fibrinogen concentrate available on the market. The product is derived from cryoprecipitate residue after extraction of factor VIII. The manufacturing process is based on a series of glycine-based precipitations—a procedure initially described by Bromback and Bromich in 1956. The cryoprecipitate extract is initially adsorbed with aluminum hydroxide, and then the fibrinogen is precipitated with high-concentration glycine. The reconstituted fibrinogen is then pasteurized. Following the addition of glycine to promote precipitation of residual proteins, a purified fibrinogen is recovered by precipitation by increasing the concentration of glycine. The pure fibrinogen precipitate is redisolved, concentrated and diafiltered, formulated, dispensed, and lyophilized. The pasteurization step is effective against enveloped and non-enveloped virus. Further viral clearance is obtained at the cryoprecipitation and glycine precipitation steps.

The EP requires that the lyophilized product be white or pale yellow, in a hygroscopic powder or friable solid; that the water content is within approval limits; and that it dissolves within 30 minutes at room temperature, forming an almost colorless, slightly opalescent solution. The solution should contain not less than 10 g/l fibrinogen with pH of 6.5 to 7.5 and a minimum osmolality of 240 mOsmol/kg. The product should be sterile and free of bacterial endotoxins.
The development of another fibrinogen product by Octapharma, called Octofibrin®, which is highly purified and incorporates two dedicated viral removal procedures (S/D treatment and viral filtration), has been reported. It is currently undergoing clinical trial.154

Prothrombin complex concentrate

The manufacture of commercially available prothrombin complex concentrates (PCC) is predominantly achieved by the use of anion exchange chromatography. Incorporated viral removal procedures include S/D treatment, heat pasteurization, vapor heat or dry heat, and viral filtration. The final formulation may involve the addition of heparin and antithrombin (ATIII) as specific clotting factor stabilizers (for the prevention of activation), and albumin as an excipient.12,155

A manufacturing process for PCC should ensure the generation of a product with a balanced content of vitamin K–dependent factors (II, VII, IX, and X) and the presence of physiologically relevant amounts of the regulatory inhibitory proteins, proteins C, S, and Z.12 The properties of various PCCs have been compared. All had similar levels of coagulation factors, but there were differences in purity and content of the inhibitory proteins.156

To specifically illustrate the principles of the manufacture of PCC, the process used for the production of Kcentra® (CSL Behring) (Beriplex® in Europe) and Octaplex® (Octapharma)—two commercial products in wide use—will be examined (Figure 27.16). The manufacture of Kcentra® is initiated by the adsorption of the PCC factors from cryoprecipitate-depleted plasma with DEAE anion exchange resin. The factors are eluted and stabilized by the addition of ATIII, heparin, and CaCl₂. The eluate is pasteurized to ensure viral safety. Further purification involves ammonium precipitation of impurities, binding to calcium phosphate, followed by elution and Aerosil® (fumed silica) adsorption of the eluate. The recovered solution is formulated by the addition of albumin, ATIII, and heparin to stabilize the active components, and then subjected to viral filtration through two 20 nm filters. Following dilution to a targeted factor IX concentration and the adjustment of the final albumin concentration, the product is dispensed and lyophilized157 (CSL information on file). In the Octoplex® process, PCC factors are adsorbed from cryoprecipitate-depleted plasma with a Q anion exchange chromatographic resin following pH adjustment and heparin addition to prevent factor activation. The recovered eluate undergoes S/D treatment for viral inactivation. Subsequent binding to a DEAE anion exchange resin allows the washing away of the viral inactivation chemicals, and provides further purification. The product is then subjected to viral filtration with a 20 nm filter. Following ultrafiltration, the product is formulated with the addition of heparin (but no albumin), then dispensed and lyophilized.12,158

Both processes generate highly a purified PCC with balanced factor content and regulatory proteins.181–183 The dedicated viral removal steps and partitioning of virus, which occur at specific process steps, ensure the viral safety of these products.159,160

There is an EP monograph for PCC products.161 It stipulates that a product must contain factor IX together with variable

![Figure 27.16 Comparison of the manufacture of two prothrombin complex concentrate products.](image)
amounts of factor II, VII, and X. The preparation may contain stabilizers (e.g., albumin, heparin, and ATIII). The lyophilized product should be a white or slightly colored, very hygroscopic powder or friable solid. Water content should be within approved limits. The preparation must dissolve completely within 10 minutes with gentle swirling. The potency of the reconstituted preparation should be not less than 20 IU of factor IX per milliliter. The pH of the solution should be between 6.5 and 7.5, and osmolality not less than 240 mOsmol/kg. If the content of any of the other factors is stated, it should not be less than 80% or more than 120% of the stated range. The specific activity of factor IX should not be less than 0.6 IU/mg of total protein (before addition of any stabilizer). The presence of any activated coagulation factors should not result in the coagulation time in a NaPTT assay to be less than 150 seconds. If heparin has been used in the formulation, the amount should comply with that on the label and not be more than 0.5 IU per IU of factor IX. The preparation must be sterile and free of bacterial endotoxins.

**Factor IX**

The approaches taken for the purification of high-purity factor IX has recently been extensively documented, and the key process stages have been collated for all the registered factor IX products.13,155 Predominantly, the process commences with the initial capture of prothrombin complex factors (factors II, VII, IX, and X). DEAE anion exchange chromatography. In the manufacture of factors II, IX, and VIII) from cryoprecipitate-depleted plasma by cryoprecipitate and involves initial adsorption of impurities from cryoprecipitate-depleted plasma by DEAE anion exchange chromatography. In the manufacture of Alphanine®, final purification involves adsorption to a barium citrate precipitate, followed by two sequential dextran sulfate affinity chromatography steps. Viral removal is achieved by S/D treatment and viral filtration.13,155 The recovery of purified factor IX in the Betafact® process involves further purification by DEAE anion exchange chromatography and heparin affinity chromatography. Viral removal is achieved by S/D treatment and viral filtration.13,163

In the Betafact® process, the crude preparation initially obtained by DEAE anion exchange chromatography, undergoes a viral inactivation procedure—pasteurization—and then is partially purified by ammonium sulfate precipitation, prior to recovery of factor IX by calcium phosphate adsorption and final purification by heparin affinity chromatography (CSL data on file). The purification of Mononine® involves passage of a DEAE anion exchange chromatography eluate, which contains PCC factors adsorbed from cryosupernatant, through a specific anti-factor IX monoclonal antibody affinity resin. The pure factor IX is eluted with sodium thiocyanate and is then diaphraged. The material is then passed through a viral removal filter. Subsequent passage through an amino-hexanoic affinity column removes any leached monoclonal antibody ligand from the previous column.164-167 The products are then formulated dispensed and lyophilized.

The required properties of a purified factor IX product are detailed by an EP monograph.168 The lyophilized product should be a white or pale yellow hygroscopic powder or friable solid. The water content should be within limits approved by the competent authority. The preparation should dissolve completely with gentle swishing within 10 minutes. The reconstituted product should have a pH between 6.5 and 7.5, and a minimum osmolality of 240 mOsmol/kg. Potency should be not less than 20 IU/mL, and specific activity not less than 50 IU/mg of total protein. Any presence of activated factors should not cause the coagulation time in the NaPTT test to exceed 150 seconds. If heparin has been used in the formulation, it should be stated on the label but in all cases should not exceed 0.5 IU of heparin per IU of factor IX. The product should be sterile and free of bacterial endotoxins.

**von Willebrand factor**

Haemate P®/Humate P® (CSL Behring) was one of the first developed intermediate-purity factor VIII–vWF products. Since its licensing in Germany in 1981 for the treatment of hemophilia A, it has also been used to this day for the treatment of von Willebrand disease.169 The manufacture of the product is from cryoprecipitate and involves initial adsorption of impurities from the reconstituted solution with aluminum hydroxide and then purification of the factor VIII–vWF complex by a series of glycine- and sodium chloride–mediated precipitation steps. Pasteurization is incorporated as a viral inactivation step155 (CSL data on file).

In the late 1980s, new purification processes were developed incorporating chromatographic procedures to improve the efficiency of manufacture, improve product purity, and facilitate the introduction of viral inactivation procedures. A detailed examination of the chromatographic approaches that can be used to purify vWF, and an overview of the manufacturing processes and the viral removal procedures utilized for registered vWF products has been published elsewhere.10,155 They show that a
range of chromatographic procedures are used consisting of anion exchange, size exclusion, and affinity chromatography. Viral removal procedures include S/D treatment, pasteurization, dry heat treatment, and viral filtration.

The purification processes and viral removal procedures for some specific vWF products are shown in Table 27.3. The starting material in all cases is cryoprecipitate. The manufacture of Alphanate® involves an initial purification involving PEG precipitation to remove impurities, followed by heparin affinity chromatography to recover the purified vWF.10,170 The manufacture of Wilate® and Wilfactin® involves the use of size exclusion chromatography and gelatin affinity chromatography respectively, to further purify the DEAE anion exchange chromatographic fraction obtained through the processing of a cryoprecipitate solution.10,171,172 The manufacturing processes incorporate S/D treatment and dry heat as viral inactivation steps. In the Wilfactin® process, viral filtration is included.

The manufacturing process for a vWF product must deliver a consistent product with respect vWF multimer composition. The EP requires that during process development, quantitative analysis of the product must be undertaken using electrophoretic and densitometric techniques to confirm that the product closely approximates the multimer distribution of a plasma reference preparation.173–175 The dispensed product must contain not less than 20 IU/ml of vWF, and the measured value must be within 20% of that stated on the label. Factor VIII must be tested if there is greater than 10 IU of factor VIII per 100 IU of vWF. The measured value must be within 40% of the stated value. vWF potency must only be measured by the ristocetin cofactor assay. vWF products are characterized on the basis of specific activity and a vWF activity–FVIII ratio. For the former, it must be greater than 1 U vWF/mg of total protein excluding any protein stabilizer. A value greater than 80 U vWF/mg protein is usual, although products with lower specific activity exist.155 The vWF activity–FVIII ratio is not prescribed but must comply with the approved limit for the product with the competent authority. Values for the various products are from 0.75 to 2.4. The exception is Wilfactin, where the ratio is approximately 60.155 This reflects the design of the manufacturing process which reduces the co-purification of factor VIII, thereby producing a predominantly vWF product.171

With respect to other product characteristics, the EP requires that the lyophilized final product be a white or pale yellow, hygroscopic powder or friable solid with a water content that is within the approved limits of the competent authority and that dissolves completely with gentle swirling within 10 minutes, giving a clear or slightly opalescent, colorless yellow solution. Some products allow for filtration to remove flakes or particles validation after reconstitution. If this is the case, it must be shown in studies that there is no impact on product potency. The reconstituted product must have a pH between 6.5 and 7.5, an osmolality greater than 240 mmol/kg, and no anti-A- or anti-B-mediated agglutination at a 1:64 dilution at a defined dilution of the reconstituted preparation. The product must be sterile and endotoxin free.175

### Factor VIII

The production of cryoprecipitate for clinical use by Judith Pool in 1965 was a pivotal event in the treatment of hemophilia as it provided a high-potency product and an alternative to what hitherto had been used-fresh frozen plasma.176 In the following years, purification processes were developed that further improved the purity and viral safety of factor VIII products. Haemate P®/Humate P®, in Germany in 1981, was the first registered intermediate-purity product containing a dedicated viral inactivation procedure. The product contained both factor VIII and vWF, and it became a recognized therapeutic for both hemophilia A and von Willebrand disease.169 The purification process increased the specific activity of vWF from 1 IU/mg of protein for cryoprecipitate to approximately 38 IU/mg of protein in Haemate P®/Humate P®,155

In subsequent years, the application of chromatographic steps further increased the purity of factor VIII products.5 Commonly referred to as high-purity products, the specific activity was typically greater than 100 IU/mg and for some products as high as 180 IU/mg.9,155 The different manufacturing processes resulted in a variation in the vWF content, which in some cases limited their use to the treatment of hemophilia A and the exclusion of von Willebrand disease. With the development of these new processes, the opportunity was also taken to introduce two specific viral removal procedures. Typically, these were S/D treatment and dry-heat treatment of the lyophilized final product. Vapor heat and viral filtration have also been applied.9,155 The use of monoclonal affinity chromatography allowed the generation of factor VIII concentrates with higher specific activity: >3000 IU/mg. These products, of course, do not have vWF.9,155

Table 27.4 presents the manufacturing process of a number of commercially available factor VIII products. The aim is to specifically present details to illustrate the range of processes that have been used to purify Factor VIII and ensure viral safety. In the manufacture of the high-purity products BeriataP® (CSL Behring), Faktane® (LFB), and Immunate® (Baxalta), there is typically an initial Al(OH)₃ adsorption step. This serves to remove vitamin K–dependent prothrombin complex factors. Further purification then

### Table 27.3 Examples of manufacturing processes for von Willebrand factor

<table>
<thead>
<tr>
<th>Brand Name (Manufacturer)</th>
<th>Purification Process</th>
<th>Specific Viral Removal Steps</th>
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</thead>
<tbody>
<tr>
<td>Haemate P®/Humate P® (CSL Behring)</td>
<td>Aluminum hydroxide treatment, Glycine precipitation, Sodium chloride precipitation, NaCl/Glycine precipitation</td>
<td>Pasteurization (60°C for 10 hours)</td>
</tr>
<tr>
<td>Alphanate® (Grifols)</td>
<td>Polyethylene glycol precipitation, Heparin affinity chromatography, DEAE anion exchange chromatography, Size exclusion chromatography</td>
<td>Solvent/detergent treatment, Dry heat (80°C for 72 hours)</td>
</tr>
<tr>
<td>Wilate® (Octapharma)</td>
<td>Alumina gel treatment, DEAE anion exchange chromatography (X2), Gelatin affinity chromatography</td>
<td>Solvent/detergent treatment, Viral filtration, 35 nm, Dry heat (80°C for 72 hours)</td>
</tr>
<tr>
<td>Wilfactin® (LFB)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

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Section II: Part IV: Plasma

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occurs by anion exchange chromatography.\textsuperscript{177–179} Factane\textsuperscript{\textregistered} has a unique viral filtration step involving passing the product through a 15 nm filter. It had been thought that the factor VIII–vWF complex was too large to be able to successfully filtered. However, this was achieved by dissociating the complex prior to filtration by exposure to calcium chloride and then reassociating the complex by its removal from the filtrate. The manufacturing process for Alphanate\textsuperscript{\textregistered} differs in that there is an initial PEG precipitation step that is effective in removing fibrinogen and fibronectin. Final purification utilizes heparin affinity chromatography.\textsuperscript{180–182}

The examples of the manufacture of very-high-purity factor VIII products offer some interesting and distinct differences. In the case of Hemofill M\textsuperscript{\textregistered}, following a cold precipitation clarification step of the cryoprecipitate suspension, the solution undergoes S/D treatment to effect viral inactivation. Factor VIII purification is achieved by anti-factor VIII immunoaffinity chromatography. This step is very effective in achieving the removal of fibrinogen, fibronectin, and vWF. There is then a viral filtration step. A subsequent Q anion exchange chromatographic step binds factor VIII eluted from the affinity column and allows any leached monoclonal antibody affinity ligand to flow through.\textsuperscript{183,184} In the Monoclate\textsuperscript{\textregistered} process, the cryoprecipitate suspension is treated with aluminum hydroxide for clarification and removal of vitamin K–dependent clotting factors. The recovered solution is then pasteurized to provide a viral inactivation step. The factor VIII–vWF complex is captured by an immobilized anti-vWF antibody. Factor VIII is dissociated with the used elution conditions, recovered, and passed over an amino hexanoic affinity column to remove any possible leached monoclonal antibody affinity ligand\textsuperscript{185,186} (CSL data on file).

All factor VIII products are lyophilized. Various excipients are used and can include amino acids, sugar alcohols, sugars, and salts. In some cases, albumin is also included.\textsuperscript{155}

The required properties of factor VIII are detailed in the EP\textsuperscript{187} The specific activity should not be less than 1 IU of factor VIII per milligram of total protein before the addition of any protein stabilizer. But, as has been noted, with modern purified products this is readily achieved. The potency of the preparation as stated on the label should result in not less than 20 IU factor VIII per milliliter. With respect to the appearance of the lyophilized preparation, water content, solubility, pH, osmolality of the reconstituted material, anti-A and anti-B hemagglutinin titer, sterility and endotoxin content, the requirements previously described for the vWF product apply.

**Conclusion**

The production of plasma protein-based therapeutic products is a complex process commencing with the collection of plasma by a system incorporating donor selection, viral testing and quality assurance measures that ensure the viral safety of the plasma for fractionation.\textsuperscript{13} Starting with the work of Cohn, and through the subsequent application of knowledge of protein chemistry, bioprocessing technology, and virology, processes have been developed for the production of therapeutic products for a range of clinical indications, which meet the high standards of safety and efficacy prescribed by the regulatory authorities.\textsuperscript{7}

Today, the production of plasma protein therapeutic products is a large global industry.\textsuperscript{31,188} Despite the development of many recombinant alternatives, the production of products by fractionation of plasma remains cost-effective and continues to grow.\textsuperscript{189} There is particular growth in the provision of albumin and immunoglobulins.\textsuperscript{190} Albumin is required in large amounts, and immunoglobulins have unique biological properties. These factors make it commercially unattractive or scientifically unjustifiable to produce these products by recombinant means, and therefore they will most likely continue to be produced from plasma for the foreseeable future.\textsuperscript{19,189,191}

Plasma fractionators are continuing to explore the clinical utility of additional plasma proteins and improve existing products. Proteins with therapeutic potential that are being investigated are shown in Table 27.1. Plasmin and reconstituted HDL are the most advanced in the clinical development pathway.\textsuperscript{19,25}

**Table 27.4** Representative manufacturing processes for factor VIII

<table>
<thead>
<tr>
<th>Brand Name (Manufacturer)</th>
<th>Purification Process</th>
<th>Specific Viral Removal Steps</th>
<th>Specific Activity (IU/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Intermediate-Purity Product</strong></td>
<td></td>
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</tr>
<tr>
<td>Haemate P\textsuperscript{\textregistered}/Humate P\textsuperscript{\textregistered} (CSL Behring)</td>
<td>Aluminum hydroxide treatment</td>
<td>Pasteurization (60°C for 10 hours)</td>
<td>38</td>
</tr>
<tr>
<td></td>
<td>Glycine precipitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Sodium chloride precipitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>NaCl/Glycine precipitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>High-Purity Products</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beriate P\textsuperscript{\textregistered} (CSL Behring)</td>
<td>Al(OH)\textsubscript{3} adsorption</td>
<td>Pasteurization</td>
<td>170</td>
</tr>
<tr>
<td></td>
<td>Al(OH)\textsubscript{3}/QAE anion exchange resin adsorption</td>
<td>Viral filtration, 20 nm</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DEAE anion exchange chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Factane\textsuperscript{\textregistered} (LFBI)</td>
<td>Al(OH)\textsubscript{3} adsorption</td>
<td>Viral filtration, 35 nm, 15 nm</td>
<td>&gt;100</td>
</tr>
<tr>
<td></td>
<td>DEAE anion exchange chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FVIII/vWF dissociation-0.3 M CaCl\textsubscript{2}</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>FVIII/vWF reassociation</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Very-High-Purity Products</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Immunate\textsuperscript{\textregistered} (Baxalta)</td>
<td>Al(OH)\textsubscript{3}, adsorption</td>
<td>Solvent/detergent treatment</td>
<td>75</td>
</tr>
<tr>
<td></td>
<td>DEAE anion exchange chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>PEG precipitation</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Heparin affinity chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Alphanate\textsuperscript{\textregistered} (Grifols)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Very-High-Purity Products</td>
<td>Recovery of cryoprecipitate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hemofill M\textsuperscript{\textregistered} (Baxalta)</td>
<td>Anti–factor VIII affinity chromatography</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>QAE anion exchange chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Monoclate\textsuperscript{\textregistered} (CSL Behring)</td>
<td>Al(OH)\textsubscript{3} adsorption</td>
<td>Pasteurization</td>
<td>~2000</td>
</tr>
<tr>
<td></td>
<td>Anti-vWF affinity chromatography</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Amino hexanoic affinity chromatography</td>
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</tbody>
</table>
With respect to existing products, the focus is on improving formulations and addressing issues that can lead to adverse reactions. The development of high-concentration immunoglobulins for subcutaneous use, ensuring that procoagulant activity is removed during the manufacture of immunoglobulins, the introduction of a procedure to remove anti-A and anti-B hemagglutinins from immunoglobulin products and the development of a liquid formulation for alpha1-protease inhibitor are examples of these activities.

Plasma manufacturers are confronted with a limited and expensive starting raw material—plasma.192 An efficient means of producing plasma-derived products that is high yielding and cost-effective would be of commercial benefit to the manufacturer and welcomed by the healthcare community, which is seeking an affordable and adequate supply of products. Recent trends in the use of disposable technologies and the developing concept of continuous processing may help to decrease the costs of establishing and operating a manufacturing facility.193–195

The developed countries are well served for fractionation capacity. Many developing countries such as China and Brazil are extending and improving their capabilities.31 However, there are many developing countries that do not have adequate access to plasma protein products and cannot afford to purchase the requirements for their population.196 Arguments for and against the establishment of a localized fractionation facility have been discussed.192,197 The adoption of improvements in bioprocessing approaches could make the design and establishment of a fractionation facility less complex, cheaper and hence feasible for construction and operation in a developing country.

The challenge for the future is aptly captured by the following reflection: “Nearly 2 billion people—or a third of the world’s population—lack access to essential medicines. We need to ask ourselves: what use is our scientific endeavour and innovation when they do not come to the aid of people who need it the most? Should a drug be described a ‘blockbuster’ by a billion-dollar label or a billion-patients label?”198

The plasma fractionation industry will continue to deliver unique therapeutics essential for human health. It is likely that the future will be as eventful as the period from Cohn to the present day.

**Key references**

A full reference list for this chapter is available at: http://www.wiley.com/go/simon/transfusion