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Immune serum globulin is in high demand, and that demand will only increase. Yet despite product shortages, the complexities involved in creating new manufacturing processes discourage change. The benefits — increased yield, reduced costs, new technologies, and faster throughput — now make identifying the best place in the processing stream to make manufacturing changes a rewarding endeavor. Planned change always trumps losing market share or being forced to change by new regulations.

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Integrating New Technology into Blood Plasma Fractionation

Plasma protein fractionation is by far the largest industry segment in global therapeutic protein manufacture. More than 500 metric tons (about 492 U.S. tons) of human serum albumin (HSA) and more than 40 tons of intravenous immunoglobulins (IVIG) are produced annually from more than 22 million liters of source and recovered plasma (although annual capacity may be as high as 34 million liters) (1). This \$6.9 billion industry supplies products to more than one million patients each year.

A Global Need Not Met

The United States represents the largest plasma collector and manufacturing base — more than 50% of the world supply of plasma. More than 70% of the world plasma product market is held by the top five commercial fractionators: Alpha Therapeutics (www.alphather.com), Aventis Behring (www.aventisbehring.com), Baxter BioScience (www.baxter.com), Bayer Biological Products (www.bayerbiologicals.com), and CSL/ZLB Bioplasma (www.zlb.com) (2). Plasma collection and fractionation is needed in highly populated and less developed parts of the world to ensure improved and global patient care.

Product shortages. The average HSA consumption in the developed world is 200–400 kg/million population, and IVIG use was at 20–40 kg of IgG/million population in 2000 (3). According to the Plasma Protein Therapeutics Association (www.plasmatherapeutics.org), the average monthly distribution of IVIG in the United States between November 2000 and October 2001 was 1,800 kg/month per million in population (4). That is almost 80 times more IVIG produced than the largest monoclonal antibody production in the same year (estimated to be about 255 kg) (5).

The “on-label” demand (indications for which FDA has approved the therapeutic) for IVIG, as well as the myriad “off-label” uses, place immense pressure on fractionators to

increase output. Baker has calculated that at a use level of 62 mg/individual per year, the global demand for IVIG would be 379 tons annually if IVIG were available to all (6). That is more than 10 times the current supply, which is considered only enough to adequately treat United States and European populations for recognized indications.

Process Changes for Better Yield

In source plasma (collected by plasmapheresis), about seven g/L of IgG is available, and processing yields are typically 2.5–4.5 g/L (although higher figures are sometimes quoted). Process optimization can possibly improve yields by another one g/L, and more extensive use of recovered plasma (from whole blood collections) would increase starting titers. But major improvements, which might increase yield to 70% or more, need to come from process changes and the implementation of high yielding unit operations.

Barriers to change. In fractionation circles, regulators still hold the philosophy that “it is the process that defines the product.” This statement is the first barrier to change. If new technologies are to be adopted and integrated, comparability protocols will need to be accepted, a subject of hot debate (7). Fractionators and regulators need to work together to pull down the barriers (8).

Plasma contains about 60 g/L of protein, of which about 57 grams (not including processing losses) are used for many therapeutic products. That makes this human material unique as a source of multiple products. That feature differentiates the design of fractionation processes from other processes that recover single therapeutic entities from microbial or transgenic source material — because any change in the unit operation’s sequence affects all products downstream of the change.

Therefore it is not surprising that the large-scale fractionators who process several million liters of plasma annually (and smaller manufacturers as well) have left the backbone of their processes unchanged.

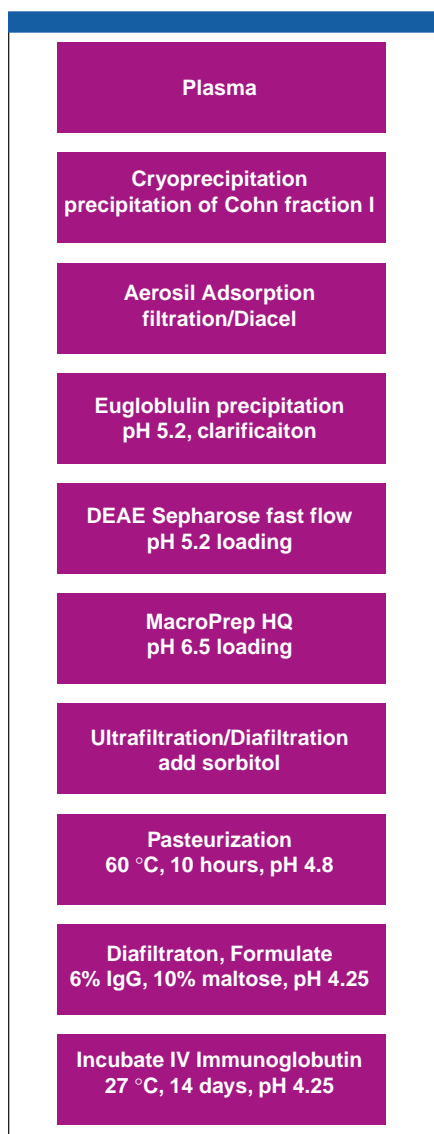


Figure 1. IVIG process developed at CSL Bioplasma, adapted from Bertolini (11); Aerosil is used as a lipoprotein adsorbent and a Diacel filter aid is used to recover the starting Cohn supernatant I before isoelectric precipitation of the euglobulin fraction at pH 5.2. The DEAE flow through is about 85% IgG, and the binding step on Macroprep HQ that follows, removes IgA.

Instead, these companies have made incremental improvements to secondary purification schemes, usually by continued processing by chromatography- and membrane-based procedures. Table 1 shows some plasma proteins of current therapeutic interest. The wide concentration range (less than one microgram per milliliter to 40 g/L) indicates one of the challenges of designing process separation procedures.

Table 1. Plasma proteins of current therapeutic interest; the wide concentration range is one of the challenges of designing process separation processes.

Protein	Concentration in Plasma	Indication
Albumin	40 g/L	Volume restoration after trauma, shock, burns
Alpha ₁ proteinase inhibitor	1.5 mg/mL	Hereditary emphysema
Anti-D IgG	titer varies ^a	Rh prophylaxis in pregnancy and childbirth
Antithrombin III	100 µg/mL	Antithrombin III deficiency
C1-inhibitor	170 µg/mL	Hereditary angioedema
Factor IX	10 µg/mL	Hemophilia B
Factor VIII	0.5 µg/L	Factor VII deficiency
Factor XI	0.3 µg/mL	Hemophilia B
Factor XIII	30 µg/mL	Factor XIII deficiency
Fibrinogen	3 g/L	Tissue sealant component
Fibronectin	300 µg/mL	Wound healing
Hepatitis B IgG	titer varies ^a	Hepatitis immunity
Immunoglobulin G	Up to 12.5 g/L	Primary and secondary immune deficiency
Measles IgG	titer varies ^a	Measles protection and treatment
Protein C	4 µg/mL	Neonatal thrombosis
Rabies IgG	titer varies ^a	Rabies risk
Tetanus IgG	titer varies ^a	Tetanus protection and treatment
Thrombin	150 µg/mL ^b	Tissue sealant component
Varicella Zoster IgG	titer varies ^a	Chicken pox protection
Von Willebrand factor	10 µg	Von Willebrand's disease

^aTiters (antibody concentrations) vary in µg/mL ranges. ^bAs prothrombin

E.J. Cohn's work as part of a war effort helped establish the fractionation industry. Following his work, HSA was first licensed on 27 August 1941, IgG (for intramuscular injection) on 8 September 1943, and the first IVIG preparation was licensed more than 20 years ago on 11 September 1981 (9).

CSL Bioplasma (www.csl.com.au) is unique in its adoption of a fractionation scheme other than Cohn–Oncley. In 1994, the company opened a new chromatography facility; in 1995, it came out with chromatographically purified HSA, and by 2000, it had a chromatography-based product portfolio.

Motivators for change. Process change is driven by market demand, improved plant throughput (output), decreased cost of goods, increased plant output and efficiency from incorporating new technologies, improved process robustness, and enhanced product safety. Improved safety can mean reduced plasma protein impurities that cause undesirable reactions, increased safety margins, or removal of human pathogens.

No other industry is quite as conscious of product safety as plasma fractionators. The industry has a substantial product safety record during the past decade. Implementing change is still a major

challenge. Still, change must come and with it will come new plasma products. Many products are currently in development: butyryl cholinesterase, apolipoprotein, active plasmin, apotransferrin, mannan-binding lectin, and fibronectin as well as proteins expressed in transgenic plants, animals, and yeast and mammalian cell culture systems (10).

The new plasma-derived products will be purified using technologies commonplace in the biotechnology industry. Yields would be high if plasma was used as a starting material, but it is likely that the main fractions of the Cohn scheme will be used as starting points.

Process Changes for IVIG

IVIG is used to treat primary and secondary immune deficiency diseases and is prepared from fraction II + III paste (the Cohn process) or precipitate A (the Kistler-Nitschmann scheme). At these stages, at least 30% of the IgG is lost, and commercial manufacturing from either of these starting points requires further liquid–solid separations after ethanol or polyethylene glycol (PEG) precipitation and after filtration, chromatography, and virus reduction steps. Commercial yields can,

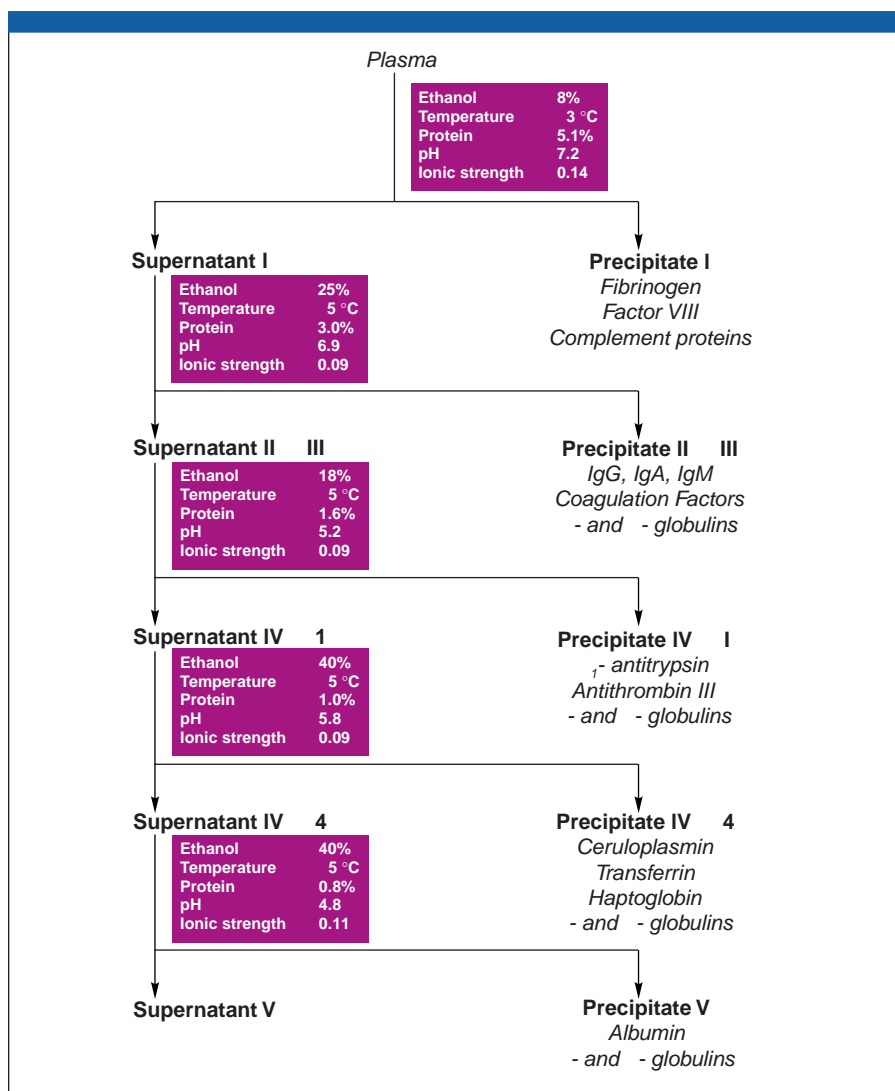


Figure 2. Basic fractionation scheme using the Cohn process (Method 6) and indicating the main protein compounds and the separation conditions for each of the five major fractions

therefore, be as low as 50% of the original IgG, leaving significant room for improvement.

What to change. What changes to the process can reasonably be made? Any change in the trunk fractionation, after cryoprecipitation and selective ion exchange or affinity adsorption of labile proteins, would be radical because it would affect every other protein manufactured at the plant. Such a change would necessitate new clinical trials and new product registration in all of the countries in which the products are licensed — a high price to pay for possible yield increases of an already safe product. Only a company without a manufacturing history, building a new facility on a “green-field” site, could afford this process change,

despite the significant advantages of modern membrane and chromatographic technologies in widespread use throughout the biotechnology industry.

Other process methods. CSL Ltd., a manufacturer that originally used the Cohn technique, opted to use chromatography. Its IVIG process starts at supernatant I, then uses sequential ion exchange chromatography on DEAE Sepharose (Amersham Biosciences, www.apbiotech.com). The process initially binds HSA then feeds the flow-through onto Macrorep HQ (Bio-Rad, www.bio-rad.com) for primary separation (11). That process continues development work done at Pharmacia in the 1970s and has taken 30 years to become a mainstay (12). A report on the continued development was reported at the 2001

Plasma Biotechnology Meeting (13). Andersson, Hübinette, and Lindquist reported on an ion-exchange process that relies on the same initial step to remove albumin, followed by two further ion-exchange steps before the solvent/detergent (S/D) virus inactivation and the removal of reactants on CM Sepharose (13).

In Figure 1, Aerosil (Degussa, www.degussa.com) is used as a lipoprotein adsorbent, and a Diacel (CFF GmbH & Co. KG, www.cff.de) filter aid is used to recover the starting supernatant before isoelectric precipitation of the euglobulin fraction at pH 5.2. Albumin is eluted from the DEAE resin and processed further to Albumex (CSL’s trade name for albumin). The DEAE flow-through is about 85% IgG, and the following binding step on Macrorep HQ removes IgA. There are two viral inactivation steps: Heat treatment at 60 °C for 10 hours at pH 4.8, and a protein concentration of 1.5%. The dispensed product is formulated at pH 4.5 and incubated at 27 °C for 14 days. Figure 2 shows method 6 of the Cohn process (14) and indicates the separation condition for five fractions and the protein components.

An older procedure developed at the Green Cross Corporation (now Mitsubishi Pharma, www.m-pharma.co.jp/e) uses Cohn fraction II, or II + III, as a starting point, employing PEG precipitation and terminal anion exchange to reduce the IgM and IgG polymer levels (15). Anion exchange steps are frequently used to reduce IgA levels, also removing remaining vitamin K-dependent coagulation factors. Prekallikrein activator (PKA) and kallikrein can be removed using affinity adsorbent with a diamino ligand such as benzamidine (16). The procedure incorporates pasteurization using sorbitol as a stabilizer.

A similar method published by Baxter BioSciences (www.baxter.com) incorporates S/D inactivation (followed by CM Sepharose chromatography), anion exchange, and PEG precipitation. This method starts with resuspended Fraction I + II + III (17). Another far simpler methodology was developed for lyophilized and liquid Vigam (Bio Products Laboratory, www.bpl.co.uk) in the UK. Fraction II is treated with DEAE Sephadex, then receives S/D treatment, oil extraction, and CM Sepharose chromatography (18).

Purification Process Changes

Annex 1 (“Types of Changes to a Manufacturing Process”) to the CPMP guidance on demonstrating comparability in human biological products acknowledges the complexity involved in changing manufacturing prices (35). The Annex lists the following purification processes that “constitutes change.”

- **Column or resin changes**, size of the column, supplier, and cleaning and storage conditions
- **Reagents**, new supplier, specifications, or replacement of raw materials
- **Purification protocol**, additions, substitutions, elimination of specific steps

- **Scale change** in downstream process
- **Facility, purification site, or equipment** change or addition

These methods all start with fractions depleted of HSA, which is probably a prerequisite in all chromatographic processing. An alternative to DEAE adsorption in the CSL procedure is to use the asymmetrical triazine derivative Cibacron blue or an engineered isomeric adsorbent (triazinyl anthraquinone/ benzene-sulphonic acid-agarose) such as Mimetic Blue SA (ProMetic BioSciences, www.prometic.com). That adsorbent step is critical in isolating albumin (Recombunin, Delta Biotechnology, www.deltabiotechnology.com, and Aventis) expressed in *S. cerevisiae* (19).

Other expression methods. Albumin expressed in the mammary epithelium of transgenic cows, whose milk also contains a low level of bovine serum albumin (BSA), can also be purified by affinity chromatography using caprylic acid as an elution agent. Even with a target of 100,000 kg/year, “Affinity chromatography can be very cost-effective, if properly optimized” (20). These methods, when adapted to appropriate plasma feed streams, quantitatively remove HSA from the feedstock in IgG manufacturing and are also useful in proteomics for sample preparation.

The staphylococcal Protein A, now engineered in *E. coli*, is an industry standard for purifying monoclonal antibodies (MAbs) from mammalian cell cultures but cannot be used for polyclonal IgG because it fails to recognize all IgG subclasses, particularly IgG₃. Protein A is, however, used in some extra-corporeal shunts to clear circulating immune complexes from the blood stream (21).

Other adsorbents. MAbsorbents (ProMetic BioSciences) are a new group of triazinyl derivatives immobilized on ProMetic’s PuraBead (a 6%, cross-linked agarose) that can be used for polyclonal antibodies and all types of monoclonal and genetically engineered antibodies (22). These synthetic adsorbents are designed for industrial use

and can be sanitized with up to 1-N sodium hydroxide and steam sterilized if required.

Figure 3 shows MAbsorbent ligand structures initially derived from modeling the phenylalanine (Phe) 132 and tyrosine (Tyr) 133 dipeptide site in the core structure of the CH2 domain of IgG. When MAbsorbents are used in a process cascade to isolate IgG, plasma is delipidated and subjected to isoelectric precipitation at pH 5.2. After readjustment to neutral pH, albumin is first adsorbed using Mimetic Blue SA, then the flow through is fed to MAbsorbent A2P. The IgG is eluted at pH 3.0, and IgM is eliminated at that point (23). Adding a flow-through DEAE Fractogel step removes any remaining IgA. This procedure can be applied to intermediate ethanol precipitates, yielding an IgG that is free of IgA and IgM from Cohn fraction II + III (24). Burton et al. have reported that the IgG could be eluted at a higher pH with the addition of polyethylene glycol (24).

Figure 4 shows the chromatography of a fraction II + III extract in which resuspended and clarified paste is loaded at pH 7 and eluted off the adsorbent at pH 6.0 in acetate buffer containing 4% PEG. The fraction is loaded directly on to the anion exchanger and is not bound. The column is washed with acetate buffer at pH 6.0 containing 1-M sodium chloride. IgG purity is equivalent to commercial standards for electrophoresis and HPLC, and the subclass distribution is conserved.

Depending on the loss of IgG to the II + III paste (or precipitate A), these adsorbent procedures can significantly improve process yields. Because the target protein is kept in solution, ultrafiltration and diafiltration steps are easily integrated as well as nanofiltration, pasteurization, and the S/D procedures used in the CSL fractionation process. Although viral inactivation needs to be demonstrated for all schemes, it is encouraging that many procedures incorporating chromatography

yield safe products (as measured by virus validation and pharmacovigilance). In addition, elution procedures are often at low pH. Adsorbent methods that start with source plasma provide an opportunity to design fractionation schemes that are tailored to patient demands for therapeutic proteins. That is in stark contrast to the “forced-fit to an existing process” approach in which yield losses in the ethanol backbone are inevitable.

Process Changes for AAT

The complex issue of plasma fractionation process change — and the locked-in-the-trunk difficulties of the process — can be illustrated in its most poignant form in the development of processes for alpha₁-antitrypsin (AAT, an alpha₁-proteinase inhibitor). This protein is effective in the treatment of hereditary AAT-deficiency (hereditary emphysema), asthma, chronic bronchitis, cystic fibrosis, and neonatal respiratory distress syndrome

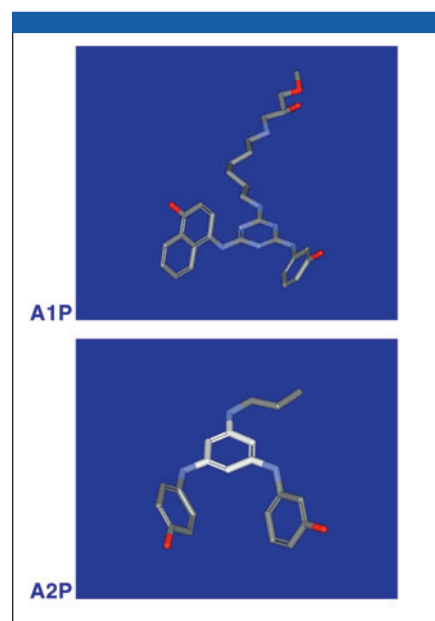


Figure 3. Ligand structures for MAbsorbents A1P and A2P (ProMetic BioSciences, Ltd.); the ligand structures were initially derived from modeling the Phe-132 and Tyr-133 dipeptide site at the core structure in the CH2 domain of IgG.

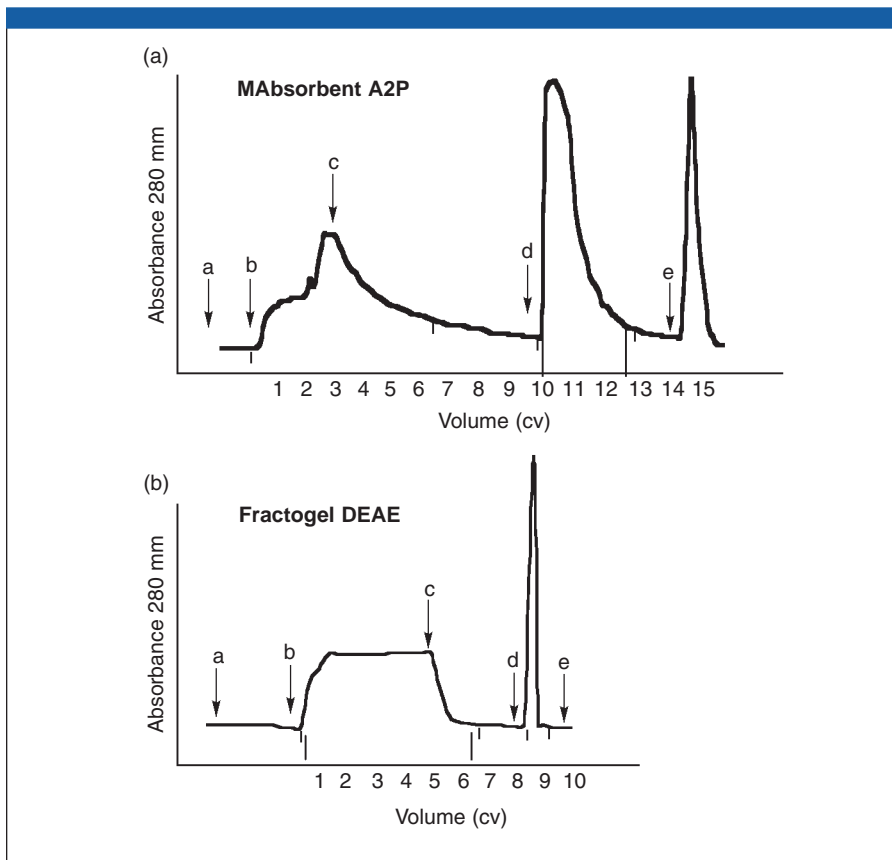


Figure 4. Chromatography of Fraction II + III on MAbsorbent A2P and Fractogel DEAE is adapted from Burton et al. (24). Resuspended and clarified paste is loaded at pH 7 and eluted off the adsorbent at pH 6.0 in acetate buffer containing 4% PEG (eluted at d). This fraction is loaded directly onto the anion exchanger and is not bound (eluted at b). The column is washed with acetate buffer at pH 6.0 containing 1-M sodium chloride. IgG purity is equivalent to commercial standards in electrophoresis and HPLC, and the subclass distribution is conserved. Courtesy of ProMetic BioSciences Ltd. (2002).

(RDS). Although licensed only for the congenital deficiency, AAT is in short supply, and ProLastin (Bayer) is distributed to AAT-deficient patients through a “Bayer Direct” program (in the United States) (25,26).

The AAT example. Besides the respiratory indications, AAT is a powerful elastase inhibitor with significant potential applications in dermatological and gastroenterological indications (now being developed by Arriva Pharmaceuticals, www.arrivapharm.com and ProMetic Life Sciences). Clearly AAT is a protein that deserves attention and which cannot be produced in sufficient quantities from plasma sources. So alternative expression systems — in *E. coli*, *S. cerevisiae*, ovine, caprine milk, and plants — are used.

AAT is currently isolated from Cohn fraction IV-1 using a process in which the starting fraction contains only 31% of the

protein (27). In the method devised by Coan et al. the paste is dissolved, subjected to fractional precipitation with PEG, DEAE-Sepharose chromatography, diafiltration, and ultrafiltration to yield sterile filtered product, which contains 50% of the starting AAT (28). The specific activity of ProLastin (the commercially available product) is ≥ 0.35 mg of functional protein/mg protein with “small amounts of other plasma proteins” (29).

The literature contains a description of the ProLastin manufacturing process (30), and in 1999, Wydick (31) described the procedure in an article on the effects of multiple product manufacturing from a single source, demonstrating the barriers to change. Although the actual processing in individual unit operations is about 36 hours, production logistics in fill-and-finish and in CBER release take 42 days and two months. Bayer is developing a new process with

anion- and cation-exchange steps, incorporating S/D treatment, reactant removal in a third cation-exchange step, and terminal dry heat treatment (32). This method improves the yield to 64%–70%, and using SDS-PAGE for purification achieves a purity of 95%.

Using biopharmaceutical techniques. The “biotechnology solution” to purification (using transgenic ovine milk) embodies the now classical capture, purify, and polish sequence and adds viral inactivation steps. The main advantage of transgenics in this case is that the expression levels in milk, which (depending on the copy number of the transgene into the genome) may be as high as 15 g/L — more than 10 times the human plasma level of 1.2 g/L (33).

Future Process Changes

Change is mandatory in the plasma fractionation industry. Its uniquely rich starting material can provide new proteins for unmet patient needs and existing proteins, particularly IgG, are likely to find new applications. Higher yields mean more revenue from the same liter of plasma. That should lead, in time, to the improvement of current facilities and the integration of new technologies into manufacturing processes. Mining the plasma proteome and developing new plasma derivatives should also increase revenue and improve earnings.

The reality of the plasma fractionator is that many process changes are being made, and many more changes are anticipated as new technologies become available. Many of these changes are driven by the industry’s safety consciousness and embody the most recent and validated procedures for virus reduction and clearance of prions. The fear of the unknown infectious agent is omnipresent, and many concerns about known pathogens are not yet fully resolved.

In the FDA guidance document on comparability, the agency clearly “recognizes that a manufacturer may seek to make changes in the manufacturing process used to make a particular product for a variety of reasons, including improvement of product quality, yield, and manufacturing efficiency” (34). The EMEA’s Committee for Proprietary Medicinal Products (CPMP) guidance is similarly positive, stating that “a flexible approach should be adopted taking into account progress made in science and technology” (35). Neither of the documents

mention plasma proteins — but neither document excludes them — and the issue of multiple products from a single source material remains to be dealt with. The complexity of changing a backbone process is evident in the detail of Annex 1 of the CPMP document, which addresses “Types of Changes to a Manufacturing Process.” Purification processes that CPMP says “constitutes change” are listed in the “Purification Process Changes” sidebar.

At a biological safety conference in 2001, Fred Feldman, then vice president and chief scientist at Aventis Behring stated that, “Safety and efficacy improvement, increase and control of supply, convenience, cost, and meeting unmet clinical needs are the potential driving forces for technology change.” Feldman went on to say, “The evolution of standards and lessons learned from early Cohn to today’s plasma therapeutic proteins set a base for migrations to other technology platforms” (36). The extent to which these new technology platforms become integrated into the main fractionation scheme remains to be seen, despite the added benefits of yield, purity, increased specific activity, and often safety. New methods will definitely be integrated into side fractionation procedures in which the process change leave unaffected other proteins, but even these smaller adjustments will require a renewed relationship between fractionator and regulatory body.

Fred Rothstein, a disciple of Cohn and a true advocate of change (read improvement), insists that process engineering, manufacturing, quality control, regulatory, and facilities engineering must be in at the start of the project and suggests that “Industry and regulatory authorities must . . . become collegial” (9).

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