2
The Use of Chromatography to Manufacture Purer and Safer Plasma Products

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Introduction

Chromatography is as old as the bible. According to the Old Testament, Moses realized that the rotten cellulose of the tree could be used to exchange the magnesium ions in the water, leaving the water sweet to taste. Centuries later, we now have a better understanding of how chromatography can be harnessed to purify not just water but one of the most precious of juices, blood.

Blood transfusion medicine can be traced back to classical Greek times when it was based on Hippocratic and Galenic concepts of four humours – sanguine, phlegmatic, melancholic and bilious. Donation from the milder species of gentle disposition was supposed to have a calming influence on the recipient of the blood. Centuries later, we have a better understanding of what makes blood so special and how to transfuse it and its derivatives to a patient in a way that is efficacious and safe.

This review looks at how chromatography came to play a key role in purifying important therapeutic products from the blood. The threat of emerging blood-borne

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Abbreviations used for products: ATIII, antithrombin III; α1PI, alpha1 proteinase inhibitor; IgG, immunoglobulin G; IgM, immunoglubumin M; PCC, prothrombin complex concentrate; RhD, Rhesis D; vWF, von Willibrand Factor.

Abbreviations used for chromatography: IEX, ion exchange; IMAC, immobilized affinity chromatography; GF, gel filtration; DEAE, dimethylaminoethyl; CM, carboxymethyl; Q/QAE, quaternary amine; S, methyl sulphonate.

Abbreviations used for viruses: BVDV, bovine viral diarrhoea virus; CPV/PPV, canine/porcine parvovirus; DHBV, duck hepatitis B virus; EMCV, encephalomyocarditis virus; HAV, hepatitis A virus; HBsAg, hepatitis B surface antigen; HCV, hepatitis C virus; HIV, human immunodeficiency virus; HSV, herpes simplex virus; MuLV, murine leukaemia virus; PRV, pseudorabies virus; VSV, vesicular stomatitis virus; BHV, bovine herpes virus; SV-40, simian virus 40.

Abbreviations used for companies: LFB, Laboratoire Français du Fractionnement et des Biotechnologies; BPL, BioProducts Laboratory; CRTS, Centre Régional de Transfusion Sanguine; CNTS, Centre National de Transfusion Sanguine.

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viruses to the use of these blood products is highlighted, and how the industry took measures to screen out these viruses, to inactivate or remove them so as to render the products safe. In particular, the promise that chromatography holds in adding safety to these products by partitioning away the adventitious blood-borne viruses will be presented. Finally, we will ponder on how best to utilize chromatography to provide clinically more efficacious and safer products for therapeutic use.

Plasma fractionation

The plasma fractionation industry as it predominantly operates today evolved from the need for a stable plasma expander to treat battlefield casualties during World War II. Cohn *et al.* (1946) developed a detailed fractionation procedure based on the manipulation of pH, ionic strength, ethanol concentration and temperature to precipitate different protein fractions from plasma. This Cohn process and modifications of the cold ethanol fractionation form the mainstay of most modern fractionation processes. The Cohn-Oncley method (Oncley *et al.*, 1949) and the Kistler and Nitschmann method (1962) are two of the most accepted methods in use today, and these are illustrated in *Figures 2.1a* and *2.1b*.

The major plasma products generated from plasma are albumin, immunoglobulin (IgG) and the coagulation factor concentrates (Factor VIII, Factor IX, and antithrombin III). However, new products continue to emerge as the functions and therapeutic values of other plasma proteins are identified (*Table 2.1*).

Factor VIII is usually isolated from cryoprecipitate and further purified by a series of precipitation steps using gentle, non-denaturing, precipitating agents such as glycine/saline and polyethylene glycol (Rock, 1991). Factor IX is generally isolated from cryoprecipitate depleted plasma using chromatography to capture the clotting factors (Dike *et al.*, 1972; Middleton *et al.*, 1973.) The source of fibrinogen is either as a discarded precipitate from the Factor VIII process or the discarded Fraction I precipitate from the Cohn process (Feldman and Winkelman, 1991). Albumin is commonly obtained from a series of ethanol precipitation steps, with the final pure albumin product obtained by precipitation with 40% ethanol. Immunoglobulin is generally produced by using 19–25% ethanol which separates immunoglobulin from albumin, and is then followed by further ethanol precipitation steps to obtain an IgG product. In order to improve safety and clinical acceptance of IgG, further processing of some IgG products is required. Such processing may involve either polyethylene glycol precipitation, ion exchange chromatography (IEX) or pepsin/low pH treatment (see a review by Knapp and Colburn, 1990). Other proteins that may be extracted during the cold ethanol process and that are used clinically include antithrombin III (ATIII), alpha1 proteinase inhibitor (α1PI), thrombin, Factor VII and C1 esterase inhibitor. These products are derived from several fractions of either the Cohn process or the Kistler/Nitschmann process, as indicated in *Figure 2.1* (*a* and *b*).

Chromatography

There are many advantages to cold ethanol fractionation, but it cannot be used in the purification of many trace plasma proteins due to its low specificity/selectivity. Chromatography, on the other hand, is recognized as a purification technique capable

Table 2.1. Current products isolated from plasma and used for therapeutic purposes

Plasma product	Preparations	Indications (Usage)
Albumin	20–25% solution 5% solution	Severe acute hypoproteinaemia Haemolytic disease of new born Shock, Burns Pump prime cardiopulmonary bypass Therapeutic plasmapheresis
Immunoglobulins (IgG)	For intramuscular injection Hyperimmune globulins For intravenous injection	Disease prophylaxis (eg hepatitis A) Agammaglobulinaemia Disease prophylaxis Prevention of sensitization ($Rh_0(D)$) Primary Agammaglobulinaemia Secondary hypogammaglobulinaemia
Factor VIII Concentrate and FVIII/ von Willibrand complex (FVIII/vWF)	Intermediate and high purity	Haemophilia A
Prothrombin complex Concentrate (PCC) and Factor IX concentrate	Intermediate and high purity	Haemophilia B Factor VII deficiency Factor IX deficiency Factor X deficiency
Antithrombin III (ATIII)	Intravenous injection	Congenital antithrombin III deficiency Thrombosis
Fibrinogen and Thrombin (Fibrin Glue)	Dual syringe kits of lyophilized products	Prevention of blood loss during surgery Mastectomy Vasectomy
Alpha 1 proteinase inhibitor (α1PI)	Intravenous injection	Hereditary emphysema Cystic fibrosis
C1 esterase inhibitor	Intravenous injection	Hereditary and acquired angioedema

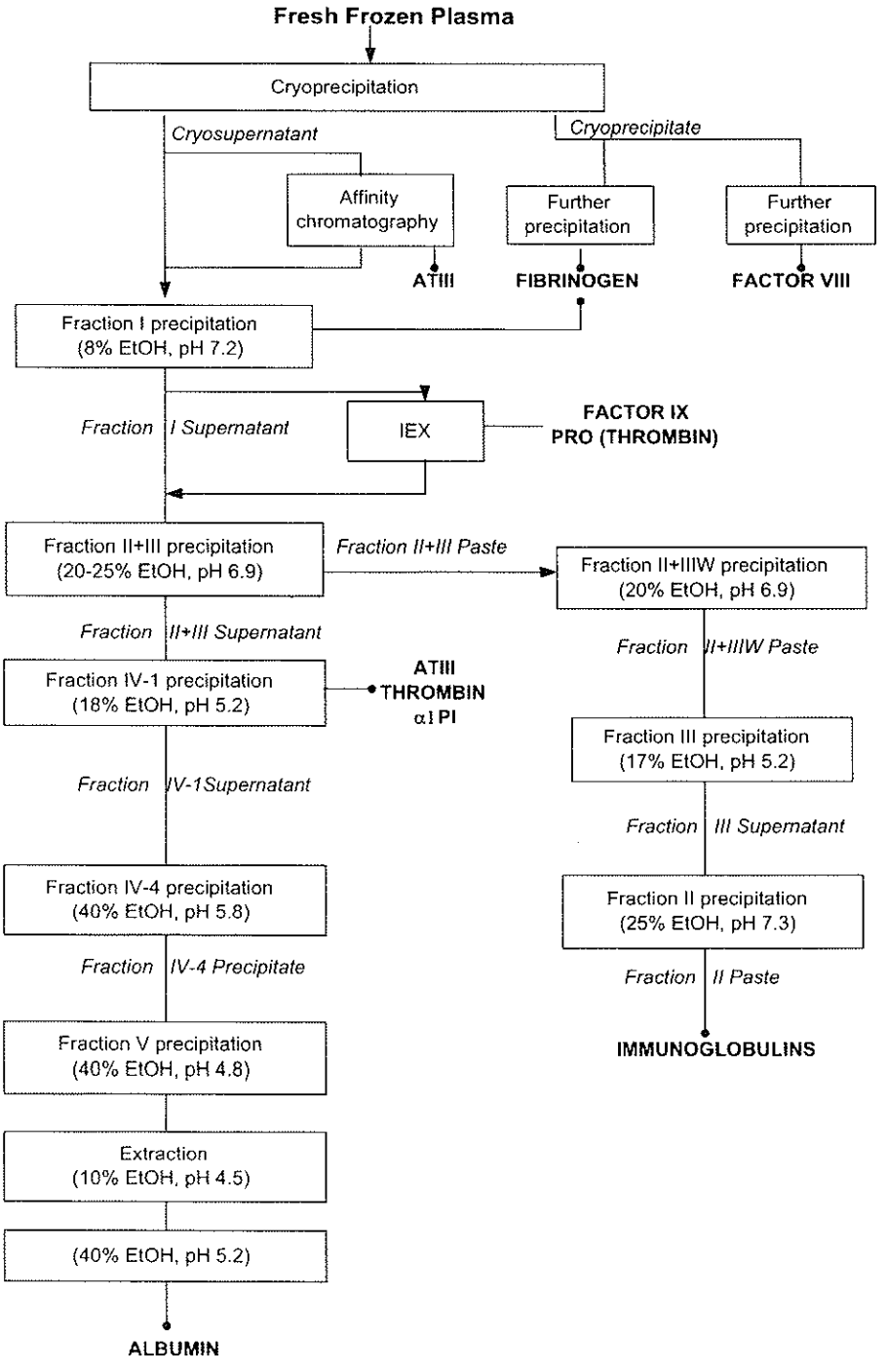

Figure 2.1a. Cohn/Oncley process.

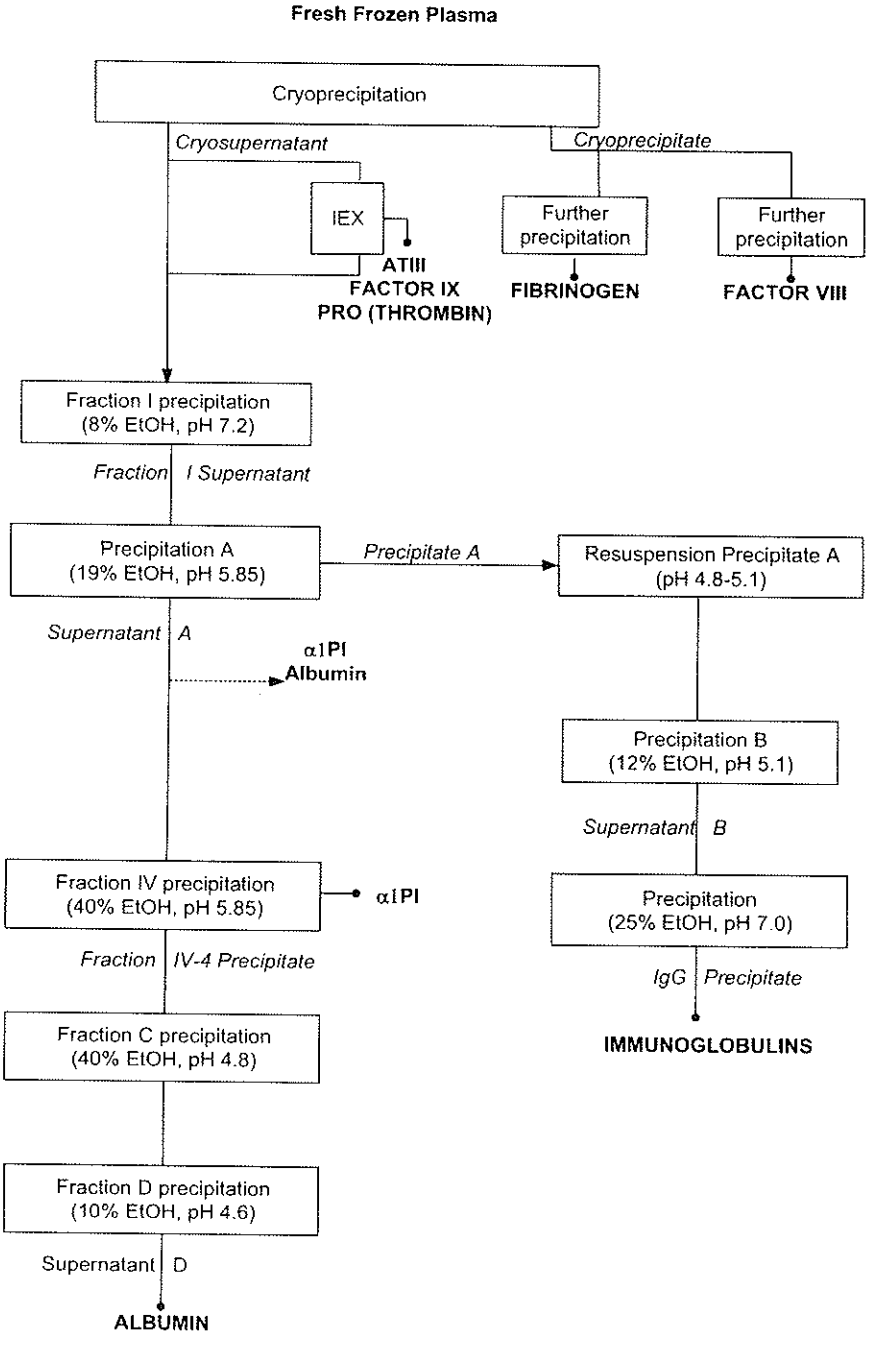

Figure 2.1b. Kistler/Nitschmann process.

of obtaining high purity products with high yields, and it is a technique that is easily automated and more versatile than precipitation.

Chromatography derived its name from its usage in the early 1900s during studies on the composition of plant pigments. Its virtues were quickly recognized in preparative work, but it was not until the 1930s that its usefulness was seen in those areas that required sensitive purification offered by few other techniques. Exploitation of its capabilities on a process-scale did not arise until Peterson and Sober (1956) developed the cellulose based ion exchange resins, which were more reliable and reproducible than their predecessors. Today, the technique of chromatographic purification has developed into a sophisticated, automated and highly tuned technique used for a wide range of therapeutic products, including those plasma products discussed in this review.

There are essentially three basic types of chromatography which are used in the following

- to remove trace protein components,
- to purify bulk target proteins from other bulk proteins,
- to capture trace proteins,
- to separate aggregates from monomers,
- to remove stabilizers, virucidal agents and salts.

Figure 2.2 illustrates the principle and demonstrates how different proteins can be separated on ion exchange, gel filtration and affinity resins. These three types, and their application in the plasma industry, are briefly discussed in the following sections.

ION EXCHANGE CHROMATOGRAPHY (IEX)

This mode utilizes ionic and surface charge differences in proteins to promote adsorption to the ionic groups such as dimethylaminoethyl (DEAE), quaternary amine (Q/QAE/QMA), methyl sulphonate (S) and carboxymethyl (CM). This is the most widely applied technique for the preparation of plasma protein concentrates for therapeutic use.

GEL FILTRATION CHROMATOGRAPHY (GF)

This mode separates proteins on the basis of differences in molecular size and architecture. Proteins or complexes do not interact with the resin; instead, separation is affected by the differential retardation of the proteins in the starting material. Gel filtration was originally used to remove salts from proteins, as described by Porath and Flodin (1959), but now has found further application in separating aggregates from dimers and monomers and larger molecular weight proteins, or complexes from smaller molecular weight proteins.

AFFINITY CHROMATOGRAPHY

Affinity chromatography harnesses a unique specificity between a ligand on the chromatographic resin and the protein of interest. With ligands such as heparin,

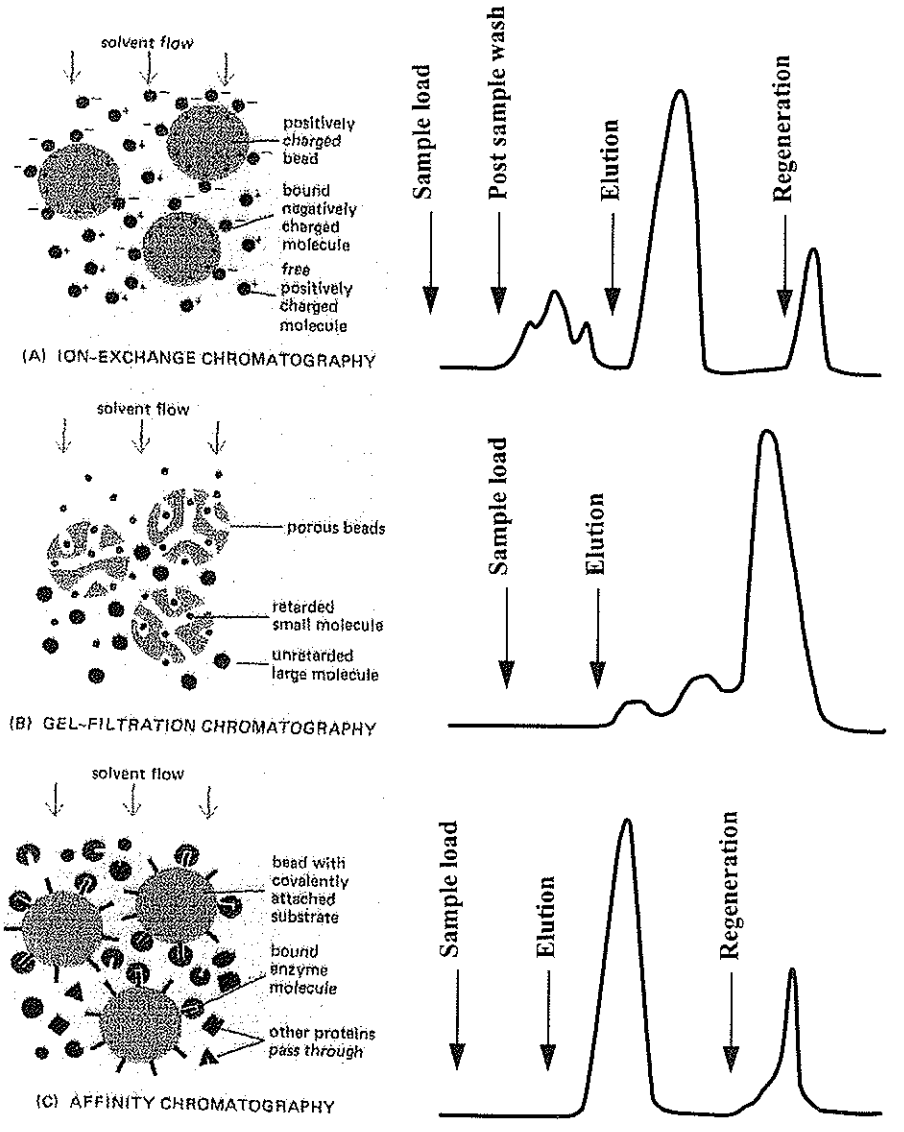

Figure 2.2. A schematic representation of three modes of chromatography.

lysine, gelatin and monoclonal antibodies, this highly selective procedure is usually used upstream to capture the target protein from the complex plasma or cryosupernatant, or used downstream as a polishing step to remove trace contaminants.

Immobilized metal affinity chromatography (IMAC) is an extremely versatile but complex separation technique. It was first developed by Porath *et al.* in 1975 and is slowly gaining recognition in the plasma industry.

Dye affinity has also been explored, principally in removing or capturing albumin from complex feeds (Birkenmeier and Koppersschlager, 1989). However, it has seen

little large-scale application owing to considerable leakage of dye and a reduction of resin capacity.

CHROMATOGRAPHIC RESINS

Considerable effort over the last fifty years has focused on improving selectivity and resolution in analytical scale operation and increasing throughput in preparative and large-scale processes. The obvious area of improvement and expansion lay in developing chromatographic resins that fulfilled the increasingly demanding requirements placed upon them during operation. Early resins consisted of polystyrene/divinyl benzene backbones to which ligands or ionic groups were attached. These early resins were strongly hydrophobic, and delicate biologicals that preferred a hydrophilic environment tended to denature upon adsorption. The cellulose resins developed by Peterson and Sober were hydrophilic and they tended to swell in aqueous environment, resulting in very large effective capacity. Peterson and Sober reported that DEAE cellulose absorbed three quarters of its weight of plasma albumin. However, today they are not widely used because of their tendencies to compress and swell during operation and cause high backpressure. The introduction of cross-linking by Porath and co-workers resulted in added mechanical strength and combated the problem of compressibility at the expense of permeability. These Sepharose and Sephadex based resins manufactured by Pharmacia (Uppsala, Sweden) are predominantly used in the plasma industry. Also used are the silica-based resins, Spherosil, Spherodex resins, which are dextran-coated silica. Newer resins still emerging that offer unprecedented capacity and flow characteristics are the macroporous resins: Macroprep, manufactured by BioRad (Hercules, CA, U.S.A.) and Porous, manufactured by PerSeptive Biosystems (Framingham, MA, U.S.A.). Good texts describing various resin alternatives can be found in the monographs of Unger (1990) and Johnston (1991).

The choice of the ligand or ionic group still remains somewhat empirical. For affinity chromatography, the ligand and its attachment are required to be strong to avoid loss of capacity during routine use and sanitation. Strong, but not irreversible, binding of the target protein to the ligand or ionic group is an important feature in adsorption chromatography, whilst non-specific binding is preferable in gel filtration chromatography.

Whilst many novel resins find use in the laboratory, not all are amenable to production-scale application. Resins have to display reproducible performance, be readily regenerated, able to withstand routine use and sanitation without losing capacity, and have good flow rate characteristics. An extensive guide to process chromatography is given by Sofer and Hagel (1997).

The following sections review the movement of chromatography into the plasma fractionation industry and to its ultimate adoption as an essential purification tool for those plasma products that are currently in the market place.

Industrial application: plasma protein purification using chromatography

FACTOR VIII

Undoubtedly, the most common preparation of Factor VIII concentration stems from

cryoprecipitation of plasma. Whilst cryoprecipitate was used for many decades for Factor VIII therapy, it was far from ideal owing to its low purity. Early endeavours at further purification focused on precipitating agents and ion exchange chromatography. The first attempts at purifying Factor VIII by ion exchange chromatography led to an unstable final product, and inhibitor formation was observed (Michael and Tunnah, 1966; Crevald *et al.*, 1961). Further trials with more rigid and higher capacity resins, such as DEAE cellulose and DEAE Sephadex A50, proved more successful. However, yields were poor, the trials were difficult to reproduce, and instability was still an issue. In the meantime, additional precipitation techniques were being explored that removed the contaminating proteins, fibrinogen and plasminogen, that were believed to cause Factor VIII instability. Today, precipitation techniques predominate as the preferred methods for Factor VIII purification; however, ion exchange chromatography is now included to remove the virucidal agents, such as solvents and detergents, which are used upstream. Examples of large-scale manufacture of Factor VIII concentrates using anion exchange media such as DEAE Sepharose FF have been reported by Burnouf *et al.* (1991) at LFB and Josic *et al.* (1994) at Octapharma (Vienna, Austria).

Recognizing the size difference between Factor VIII-vWF complexes (several million Daltons) and the major contaminates, fibrinogen and fibronectin (340 and 440 kDaltons, respectively), Thorell and Blomback (1984) investigated gel filtration as an alternative to precipitation. Using an intermediate purity Factor VIII solution as a feed, these authors demonstrated complete removal of fibrinogen and fibronectin after passage through Sephacryl S1000. Later, Dengler *et al.* (1990) reported the use of Sephacryl S400 as a means of removing solvent and detergent, and they found that there was also an increase in the purity of the intermediate precipitate by 3,000-fold.

In search of greater yields, Stender and Kaersgaard (1997) from HemaSure A/S (Gentofte, Denmark) used plasma as an alternative to cryoprecipitate as a source of Factor VIII. By directly loading plasma onto a gel filtration resin, they were able to separate Factor VIII from albumin and IgG. Ion exchange chromatography (Q Sepharose and then S Sepharose) was then used to further purify Factor VIII.

Affinity purification of Factor VIII was also gaining in recognition as a powerful and selective technique in the 1980s (Fulcher and Zimmerman, 1982; Zimmerman, 1988). By the late 1980s, the cost of producing monoclonal antibodies, and issues such as ligand leaching and media instability, were diminishing making immunoaffinity chromatography an attractive large-scale proposition. Schreiber *et al.* (1989) described the large-scale manufacture of Factor VIII using monoclonal antibodies bound to rigid chromatographic resin. These monoclonal antibodies were directed against Factor VIII-vWF complexes and captured Factor VIII from an intermediate fraction of the Cohn process. Rock (1991) also described the large-scale manufacture of Factor VIII using a monoclonal antibody directed against vWF to separate Factor VIII from vWF. Final concentration and polishing occurred on an aminohexyl agarose resin. This is the process used at Aventis Behring (formerly Centeon) (Marburg, Germany).

Heparin affinity chromatography has also been reported in the production of a high purity Factor VIII made by Alpha/Instituto Grifols (Barcelona, Spain) (Smith *et al.*, 1997).

FACTOR IX

Current methods for the preparation of intermediate purity Factor IX (PCC) are based on the methods originally described by Dike *et al.* (1972) or Middleton *et al.* (1973) and utilize anion exchange chromatography. Factors II, IX and X, amongst others, are adsorbed batchwise to a DEAE resin (eg DEAE Sepharose, DEAE cellulose) and elution occurs by column chromatography. Non-retained proteins are separated from the resin by centrifugation. This process achieved purification factors of 100- to 200-fold (Michalski *et al.*, 1993).

There have been a number of reports of thrombotic complications associated with the use of these prothrombin complexes, including thrombosis and disseminated intravascular coagulation. The mechanism of prothrombin complex induced thrombogenesis has not yet been fully elucidated; however, Factors II and X have been implicated and, therefore, a higher purity Factor IX has been required for use in major surgery. Methods for the separation of the Factor IX from Factors II and X take advantage of the differential adsorption properties of these factors to chromatographic resins. Some early examples include work of Modi *et al.* (1984), who purified Factor IX using anion exchange (DEAE) chromatography, followed by dextran sulphate (affinity) chromatography and then heparin Sepharose chromatography, and Andersson *et al.* (1975), who used the cascade ion exchange, heparin and gel filtration. Large-scale manufacture of high purity Factor IX using the two-stage approach (anion exchange chromatography coupled with heparin affinity chromatography) is now in routine production at a number of fractionators. Koenderman *et al.* (1993) described the production process at CLB (Amsterdam, The Netherlands) which utilized DEAE Sephadex adsorption followed by DEAE Sepharose FF to remove the solvent and detergent agents and trace amounts of Factor VII. The final affinity step (heparin Sepharose CL-6B) was added to remove Factors II and X. A similar process used by CSL Limited (Melbourne, Australia) has been described by Johnston *et al.* (2000a).

Newly developed affinity techniques other than heparin affinity are being adopted to capture Factor IX from a complex feed material. Feldman *et al.* (1994) described the large-scale production of high purity Factor IX using ion exchange chromatography (using DEAE Sepharose) followed by IMAC (copper chelating Sepharose). Immunoaffinity chromatography, using immobilized mouse monoclonal antibodies, was investigated by Liebman *et al.* (1985), Smith (1988) and Lutsch *et al.* (1993). Production of clinical grade Factor IX by Alpha Therapeutic Corporation (Los Angeles, CA, U.S.A.) using immunoaffinity chromatography has been reported by Feldman *et al.* (1995) and Kim *et al.* (1992).

FIBRINOGEN

Fibrinogen fractionates into both the cryoprecipitate and the Fraction I precipitate of the cold ethanol process (*Figures 2.1a* and *2.1b*). Methods for further purification commonly employ differential precipitation using several agents, such as polyethylene glycol, aluminium hydroxide, and glycine/saline salts. Fibrinogen thus prepared has been used clinically for more than a decade (Fuhge *et al.*, 1987; Burnouf-Radosevich *et al.*, 1990). Introduction of additional viral inactivation steps and the need for

stronger fibrin glues has led to the development of purer and safer fibrinogen concentrates. Today, further purification is commonly carried out by chromatographic techniques. In the process described by Tse *et al.* (1995) and adopted by the American Red Cross (Rockville, MD, U.S.A.), purification from a precipitated fraction was achieved using lysine Sepharose 4B to remove the contaminant, plasminogen, which causes degradation of fibrinogen. After several steps, including solvent detergent treatment, an anion exchange step (DEAE cellulose) was introduced to remove the unwanted virucidal agents and the residual thrombin inhibitor, which was added upstream. Burnouf in a review (Burnouf, 1991) also described the process adopted at LFB (Les Ulis, France) to produce a fibrinogen (for surgery) which used DEAE Fractogel 650M resin as the bulk purification step, then heparin Sepharose CL-6B resin to purify away the residual proteins and solvent and detergent agents to yield a 90% pure preparation.

ANTITHROMBIN III

Antithrombin III was the first plasma product purified by affinity chromatography as reported by Miller-Andersson *et al.* (1974). Large-scale processes were soon developed using either Fraction IV-1 as the starting material, cryosupernatant or Supernatant II+III. With the introduction of pasteurization to inactivate viruses, further purification was required to remove the added stabilizers and non-heparin binding proteins, as well as protein aggregates that were formed as a result of pasteurization. A second affinity chromatography step was adopted by the plasma fractionation arm of Bayer AG (Clayton, NC, U.S.A.) (Hoffman, 1989) and Instituto Grifols (Barcelona, Spain) (Biescas *et al.*, 1998) to isolate the active antithrombin III, whilst gel filtration was adopted by Bio Products Laboratory (BPL, Elstree, Hertfordshire, U.K.) to remove the stabilizers (Wickerhauser *et al.*, 1979).

THROMBIN

The conventional method for the commercial preparation of thrombin involves the incubation of prothrombin complex fraction, purified prothrombin or Cohn Fraction III with thromboplastin (or snake venom containing Factor Xa) and calcium ions (Fenton *et al.*, 1977; Denson, 1969; Esmon *et al.*, 1974). Further purification using heparin affinity chromatography to remove inactivated prothrombin, activation fragments, and other coagulation factors (eg Factor X, protein C and Factor IX) was first developed by Nordenman and Bjork (1977) and has been adopted in the manufacture of thrombin for clinical trials (Feldman and Winkelman, 1991). Oates *et al.* (1997) at CSL Limited included a gel filtration step to remove a trace protein from their thrombin process. Proba *et al.* (1996) described an improved purification scheme based on the cation exchange chromatography, and this is now the proposed process at the Scottish National Blood Transfusion Service (SNBTS, Edinburgh, U.K.) (McIntosh *et al.*, 1998).

ALBUMIN

Apart from the ethanol fractionation processes described in *Figures 2.1a* and *2.1b*,

which predominate in the fractionation industry, the most widely used process for the large-scale manufacture of albumin is based on the chromatographic steps developed by Curling (1980). This chromatographic process entails purification of albumin from pretreated plasma or Fraction II+III supernatant. First, anion exchange chromatography is used to remove IgG (if the starting material is pretreated plasma), macroglobulin, α1PI, followed by cation exchange chromatography to remove trace proteins such as transferrin, and then gel filtration chromatography to separate out the aggregates from monomeric albumin. The largest fractionator to employ this technique is CSL Limited (Yap *et al.,* 1993). Several smaller organizations, such as the South African Blood Transfusion (Johannesburg, South Africa) (Marrs, 1993), and the Institute of Blood Transfusion (Skopje, Macedonia) (Damesvska *et al.,* 1999) have also adopted chromatography. Several smaller plants that are no longer operational, such as HemaSure A/S and Winnipeg (Friesen, 1987), also applied this method. Other combinations of cold ethanol and chromatography have been published in the literature (Véron *et al.,* 1993; Tanaka *et al.,* 1998)

IMMUNOGLOBULIN (IgG)

IgG is still predominantly made by cold ethanol precipitation worldwide. Initially, chromatography was employed largely for 'clean-up' purposes, such as the removal of aggregates and enzymes (see the review of Barandun and Isliker, 1986). Briefly, Habeeb and Francis (1977) described the application of DEAE cellulose to remove aggregates of gammaglobulin and other non-IgG proteins from Cohn Fraction II. This process was combined with affinity chromatography on lysine-Sepharose to remove plasmin and plasminogen. Hoppe *et al.* (1973) used anion exchange chromatography (DEAE Sephadex) to prepare hyperimmune IgG preparations with low anti-complementary activity.

In a push for more efficient processes, greater yields and purer products, and as newer fractionation plants are being built, chromatographic techniques today are being adopted as a supplement to, or as substitute for, cold ethanol process. Condie (1979) and Björling (1985) both described chromatographic processes for the recovery of IgG from plasma. However, the IgG concentrates lack some subclass activity and are therefore not ideal candidates for clinical material. The process used to manufacture a hyperimmune IgG, RhD IgG, was described by Bees *et al.* (1989). Based on two anion exchange processes (DEAE Sepharose and DEAE Biogel) the process in Canada yielded a pure IgG, and the product thus distributed by Aventis and Nabi (Calgene, Canada) is well tolerated and used extensively in the prevention of RhD sensitization. From Fraction II+III paste as the starting material, Tousch *et al.* (1989) used a gel filtration resin for desalting, followed by an anion exchange resin (DEAE Trisacryl) to purify IgG from IgM and albumin yielding a 98% pure IgG. Similarly, Lebing *et al.* (1999) developed IgG purification from Fraction II+III using a strong anion exchanger followed by a weak anion exchanger, with the impurities being captured on the resins. Bertolini *et al.* (1998) at CSL Limited have departed even further from the Cohn process and have developed IgG from pretreated Supernatant I using two anion exchangers, again in non-binding conditions. Likewise, the scheme outlined by Bergloff and Eriksson (1984) entailed preparing IgG directly from plasma using ion exchange chromatography as an initial bulk purification.

Dam *et al.* (1999) at HemaSure A/S described an IgG that was purified from plasma using gel filtration followed by two anion exchange steps.

ALPHA 1 PROTEINASE INHIBITOR (α1PI)

Isolation of α1PI from the discarded Fraction IV-1 was first considered by Glaser *et al.* (1975) after evidence showed increased incidences of emphysema with individuals with hereditary α1PI deficiency. Early purification of a concentrate was attempted using chromatographic techniques such as ion exchange, gel filtration, affinity chromatography, thiol disulphide exchange (Laurell *et al.*, 1983) and dye affinity chromatography to remove albumin (Travis and Pannell, 1973). These techniques were not amenable to large-scale production due to low yields, low purity and leaching of ligands. The first clinical concentrate was prepared by purely precipitation methods; however, this was only 5% pure (Gadek *et al.*, 1981).

Today, α1PI is routinely purified from Fraction IV-1 paste by partial precipitation followed by an ion exchange chromatographic step included to remove low levels of albumin, lipoproteins and, in some cases, denatured α1PI. The first production-scale process based on these principles was developed by Bayer (Coan *et al.*, 1985), who currently are the sole providers of plasma derived α1PI. Alpha Therapeutic Corporation (Los Angeles, CA, U.S.A.) have also made material for clinical trials using initial precipitation steps, followed by an ion exchange process (Hwang *et al.*, 1998). Chen *et al.* (1998) have further developed the process into a totally chromatographic method from Fraction IV-1 paste which entails three ion exchange steps (DEAE Sepharose FF, Macroprep HS, Macroprep HS), the third step being included to remove any remaining contaminants, such as solvent, lipids and denatured α1PI.

In order to increase yields, Burnouf *et al.* (1987) developed a process at CNTS (now LFB) using an intermediate fraction derived from Supernatant A of the Kistler/Nitschmann process. Ion exchange chromatography (DEAE Sepharose CL-6B) was primarily employed to remove transferrin, albumin and IgG, whilst gel filtration (Sephacryl S 200) was used to remove high molecular weight proteins such as macroglobulin and haptoglobin.

SUMMARY

The examples cited above show that there is a growing usage of chromatography in the manufacture of plasma products. The versatility of this purification technique has meant that it is used in the following:

to obtain pure products by removing	Factor II and X from Factor IX
	IgG from albumin
	fibrinogen from Factor VIII
to produce stable products by removing	plasminogen from Factor VIII
	plasminogen from IgG
	aggregates from albumin monomer
to produce clinically acceptable products by removing	
	aggregates from IgG
	denatured antithrombin III from native antithrombin III
	denatured α1PI from native α1PI

The next sections will illustrate that this powerful technique also has the capability of adding safety to already pure products by contributing to the removal of viruses during the manufacturing processes.

Blood-borne viruses

Plasma proteins, whilst giving therapeutic benefit to many, also carry the risk of transmitting blood-borne diseases to the recipients. Transmission of viral diseases by protein solutions derived from human blood plasma has been recognized for over 40 years, and examples are cited in reviews by Cuthbertson *et al.* (1991) and Horowitz and Piet (1986).

Only a few viral pathogens have been associated with blood transfusion, and fewer still associated with therapeutic proteins prepared from human plasma pools. The blood-borne viruses of greatest concern are those which cause hepatitis and auto-immune deficiency syndrome (AIDS). These viruses are hepatitis A virus (HAV), hepatitis B virus (HBV), hepatitis C virus (HCV – the major non-A, non-B hepatitis virus) and human immunodeficiency virus (HIV). Other viruses that are emerging and that are known to be transmitted by blood and/or plasma products include hepatitis G virus and human parvovirus (B19).

Transmission of viruses by plasma products

COAGULATION FACTORS

Of the manufactured plasma derivatives, the coagulation factor concentrates (Factor VIII, vWF, Factor IX and prothrombin complex) have had the highest associated risk of transmitting viruses through transfusion (Gerety and Aronson, 1982; Barker and Hoofnagle, 1974). Prior to 1985, and in the absence of any direct test for non-A, non-B hepatitis, most haemophiliacs who received clotting factor concentrates for the first time developed hepatitis C, formally known as non-A, non-B hepatitis (Fletcher *et al.*, 1983; Kernoff *et al.*, 1985). As with HCV, the incidence of transmission of HBV by Factor VIII and prothrombin complex to haemophiliacs was also very high (Scaroni *et al.*, 1980) and dropped dramatically with the introduction of HBsAg screening. Haemophiliacs were also at high risk for infection with HIV (Curran *et al.*, 1984) such that, by the mid-1980s, 50–90% of haemophilia A and B patients had developed antibodies to HIV (Jason *et al.*, 1986). The introduction of solvent/detergent treatment and dry heat treatment during the mid-1980s, together with improved viral screening methods, have meant that the risk of transmission of enveloped viruses by coagulation factor concentrates is no longer significant (Skidmore *et al.*, 1990; Rizza *et al.*, 1993; Morgenthaler, 1989). Transmission of HAV to blood product recipients prior to 1988 had only ever occurred rarely. However, in the following six-year period a number of cases were reported from Italy (Mannucci, 1992), Germany (Gerritzen *et al.*, 1992), Ireland (Temperley *et al.*, 1992), Belgium (Peerlinck and Vermylen, 1993) and the United States (McCarthy, 1996). All these cases were associated with the use of a solvent/detergent treated Factor VIII product purified by ion exchange chromatography. In 1995, the first case of HAV being transmitted by a Factor IX concentrate manufactured using a solvent detergent method of viral inactivation was reported.

There have been a number of reports of transmission of B19 virus by Factor VIII concentrates (Prowse *et al.*, 1997) which is supported by a high antibody positive rate to B19 in haemophiliacs compared to the general population (Matsunaga, 1995).

IMMUNOGLOBULINS

Immunoglobulins, prepared by cold ethanol fractionation for either intramuscular or intravenous administration, have had a remarkable safety record. Intramuscular immunoglobulins, subsequent to the inclusion of sensitive third generation tests screening for HBsAg, have not been reported to transmit viral diseases, including HBV, HIV and HCV. As with intramuscular products, intravenous immunoglobulins (IVIG) have not been reported to transmit neither HIV nor HBV since screening. However, there were a number of reports of HCV (non-A, non-B) transmission before the screening of plasma for HCV (anti-HCV) in 1990 (Lane, 1983; Ochs *et al.*, 1985; Bresee *et al.*, 1996). Further work has indicated that neither cold ethanol fractionation nor the use of plasma negative for anti-HCV is sufficient to eliminate the incidence of HCV transmission (Pawlotsky *et al.*, 1994; Yei *et al.*, 1992). Hence, manufacturers of IVIG products today include a validated method of virus inactivation (such as solvent/detergent or pasteurization) in the manufacturing process.

ALBUMIN

Albumin, prior to the introduction of pasteurization in the 1940s, had been reported to transmit serum hepatitis (Gellis *et al.*, 1948). Albumin prepared using cold ethanol fractionation *and* pasteurization of albumin in solution at 60°C for 10 hours has not been associated with viral transmission (Cuthbertson *et al.*, 1991; Roberts, 1996a; Finlayson, 1979).

Virus inactivation and removal

Unfortunately, there is no way in practice to prove the absolute freedom from viral contamination of a given product. Therefore, the risk assessment relies completely on the available data on: (i) the frequency of window period donations; (ii) virus titres of these donations; and (iii) the extent of virus reduction/inactivation anticipated in the manufacturing process (from virus validation studies). Several texts and guidelines are available to ensure quality is met (CPMP/268/95, Grun *et al.*, 1992; EPFA, 1995).

Viruses used in validation studies may either be the viruses of concern (relevant virus) in human plasma or viruses non-pathogenic to humans but which have similar physiochemical properties to the relevant viruses (model viruses). It should be kept in mind, however, that all viruses used in validation studies are model viruses as laboratory strains of even the human blood-borne viruses can differ from the strains prevalent in the circulating blood (CPMP/268/95). Examples of viruses used by fractionators are given in *Table 2.2*.

Specific virus inactivation steps have been included in each process to enhance the safety of the products by reducing the virus load during manufacturing. These steps are well reviewed by Eibal *et al.* (1988) and Roberts (1996a).

Other process steps that are considered to contribute to the overall removal of

Table 2.2. Examples of principal viruses that have been used in virus validation studies

Viruses	Genome	Size (nm)	Envelope	*Family*	Comments
HIV-1	RNA	80–100	Yes	*retroviridae*	Used as a *relevant* virus in preference to the model virus sindbis
Murine leukaemia virus (MuLV)	RNA	80–110	Yes	*retroviridae*	Commonly used as a *model* for HIV
Sindbis virus	RNA	60–70	Yes	*togaviridae*	Commonly used as a *model* for HIV. However, sindbis exhibits higher thermal and solvent detergent stability
Hepatitis B virus (HBV)	DNA	42–47	Yes	*hepadnaviridae*	Hepatitis B surface antigen (HBsAg) used as a *marker* virus in some partitioning studies HBV DNA also used
Duck hepatitis B virus (DHBV)	DNA	40–50	Yes	*hepadnaviridae*	DHBV is similar in structure to HBV and regarded as a good *model*. Used as a *model* for HBV
Herpes simplex virus (HSV)	DNA	100–150	Yes	*herpesviridae*	Used as large DNA viruses sometimes used as *model* for HBV
Pseudorabies virus (PRV)	DNA	120–200	Yes	*herpesviridae*	
Hepatitis A virus (HAV)	RNA	27–32	No	*picornaviridae*	Used as a *relevant* virus in preference to the model virus EMC or polio virus
Polio virus	RNA	25	No	*picornaviridae*	Polio virus is similar in size and structure to HAV and regarded as a good *model*
Encephalomyocarditis virus (EMCV)	RNA	25–30	No	*picornaviridae*	EMCV is similar in size and structure to HAV and therefore used as a *model*
Hepatitis C virus (HCV)	RNA	~30–60	Yes	*flaviviridae*	HCV is yet to be cultured
Bovine viral diarrhoea virus (BVDV)	RNA	50–70	Yes	*togaviridae*	BVDV is currently regarded as the best *model* virus for HCV
Human parvovirus (B19)	DNA	18–24	No	*parvoviridae*	Human B19 is not readily grown or assayed *in vitro*
Canine parvovirus (CPV)	DNA	18–26	No	*parvoviridae*	CPV is considered a good *model* for B19

viruses are precipitation steps, such as the cold ethanol process steps (Johnston *et al.*, 1996; Louie *et al.*, 1994; Hénin *et al.*, 1988), or the glycine/sodium chloride precipitation step in the Factor VIII process (Hilfenhaus and Nowak, 1994). Microporous viral filters (referred to as nano filters) are also growing in popularity since they have the potential to remove the small viruses such as HAV and B19 from plasma proteins, such as Factor IX (Hamamoto *et al.*, 1989; Burnouf-Radosevich *et al.*, 1994; Hoffer *et al.*, 1995). In the early 1990s, studies were reported showing that chromatographic steps could also contribute to virus removal. These studies come more than a decade after published work on the use of chromatography to purify viruses from plasma (Einarsson *et al.*, 1978; Einarsson *et al.*, 1981), cell culture (Chudzio and Inglot, 1973; Krasilnikov *et al.*, 1981), and other extracts (Hjort and Moreno-Lopez, 1982).

The following sections review the studies reported to date on the partitioning of viruses during the manufacture of several plasma products.

VIRUS PARTITIONING USING CHROMATOGRAPHY

As validation studies cannot be conducted in the production plant (and therefore are generally not at production-scale because of the obvious undesirability of contaminating the production facility), the first requirement of these studies is to scale-down the process. Ensuring the validity of the small-scale chromatographic process to mimic the full-scale process in the production facility is usually achieved by comparing chromatograms and the purity of eluates from both scales (Grun *et al.*, 1992). Virus studies are then performed by spiking the starting material of the manufacturing process with a high titre virus stock and then evaluating the titre of virus in the peaks

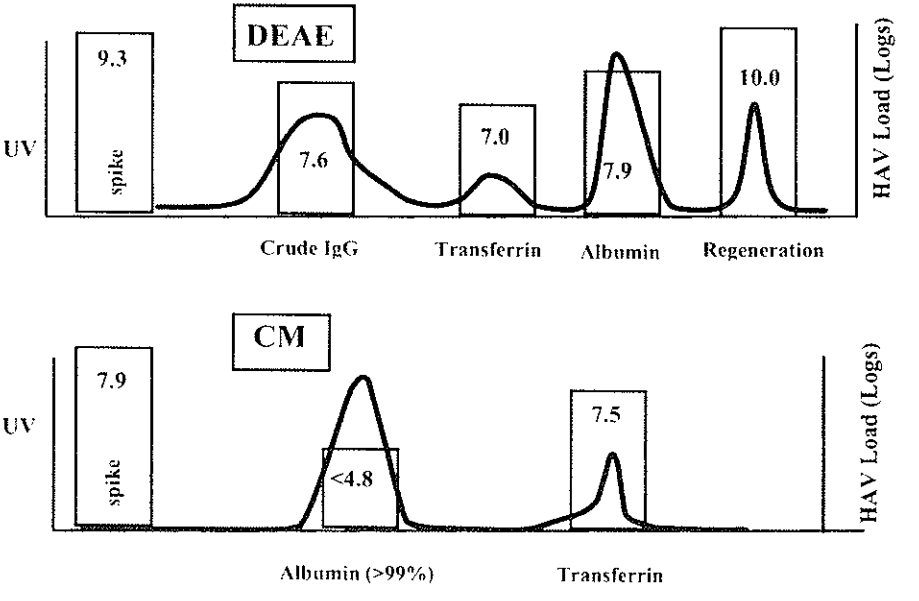

Figure 2.3. HAV partitioning during IEX chromatography of pretreated Supernatant I.

produced from the purification step. A typical outcome is illustrated in *Figure 2.3*, showing the chromatographic profile of albumin purification from pretreated Supernatant I overlaid with the viral load of HAV obtained for each major peak (Adcock *et al.*, 1999).

Similar validation studies have been carried out by several fractionators as part of their risk analysis. Published data are summarized in *Tables 2.3* to *2.7*. In the context here, and as described in the regulatory guidelines (CPMP/268/95), a step is considered an 'effective virus inactivation/removal step' if the log reduction (defined as the log viral load before the step – the log viral load after the step) is greater than or equal to 4. Similarly, the guidelines regard a log reduction less than or equal to 1 as not significant, implying that log reduction factors from = 1 to = 4.0 are recognized as useful contributors to product safety.

FACTOR VIII

Introduction of chromatographic steps to the manufacturing processes of Factor VIII concentrates has led to a significant (and efficient) overall reduction of viral load across all steps in the majority of cases. In some individual steps, the results are not so comprehensible.

In the study of Smith *et al.* (1997), a reduction of 7.6 logs for BHV (their model virus for HBV) was demonstrated over a heparin affinity step. In striking contrast, they also showed no reduction of the other viruses studied (polio virus, CPV and BVDV).

Of the weak anion exchange chromatographic steps, only the EMCV results in the study of Mohr *et al.* (1994), and the PRV results in the study of Burnouf-Radosevich and Burnouf (1992) showed a log reduction factor of > 4.0. The two studies on the strong anion exchange resins, that of QAE Sepharose (Lawrence, 1993) and Q Sepharose (Kaersgaard, 1996) gave consistent results of 2–3 log reductions for EMCV, sindbis virus, vesicular stomatitis virus (VSV), polio virus and bovine herpes virus (BHV). All other results listed in *Table 2.3* appeared without trend or pattern.

The two studies on gel filtration obtained from Kaersgaard (1996) and Mitra *et al.* (1994) were in conflict, either as a result of each study using different starting materials, or the fact that no two viruses were identical. In the presence of IgG and albumin, Kaersgaard (1996) observed no significant log reduction of BHV, BVDV and CPV, whilst in the absence of IgG and albumin, Mitra *et al.* (1994) showed 2–3.6 log reduction of HAV.

The results look more promising where the chromatographic steps are combined. Lawrence (1993) showed that the combination of a monoclonal antibody affinity step with an ion exchange step resulted in a log reduction of > 11 for EMCV, of 6.3 for sindbis virus, and of 4.4 for VSV. Schreiber *et al.* (1989) also showed a log reduction of 4.2 for sindbis virus, a log reduction of > 5.5 for PRV and of 4.1 for HIV over a two step chromatographic process. Finally, Kaersgaard (1996) similarly showed overall log reductions of 4.6 for polio virus, 3.0 for VSV and 4.5 for BHV. In these cases, chromatography would be considered an efficient mechanism for virus removal.

FACTOR IX

Of the studies collated in *Table 2.4*, only the metal chelate affinity step of Roberts

Table 2.3. Virus partitioning over chromatographic steps in Factor VIII manufacturing processes

Chromatographic step	Log reduction ($log_{10}TCID_{50}$)	Company	Reference
Heparin agarose	7.6 for HBV 2 for HIV < 1.0 for polio virus < 1 for BVDV < 1.0 for CPV	Alpha/Instituto Grifols (Barcelona, Spain)	Ristol *et al.* (1996) Smith *et al.* (1997)
Monoclonal antibody against vWF	2.8 for sindbis virus > 5.5 for PRV 3 for HIV 1.2 for VSV	Aventis (Marburg, Germany)	Schrieber *et al.* (1989)
Aminohexyl agarose	1.8 for sindbis virus 0.9 for VSV 1.1 for HIV		
Monoclonal antibody affinity	6.9 for HAV	Baxter (Los Angeles, CA, U.S.A.)	Gowda *et al.* (1994)
Q Sepharose	0.2–2.2 for HAV		
Monoclonal antibody affinity	8.3 for EMCV 4.3 for sindbis virus 2.4 for VSV	Baxter	Lawrence (1993)
QAE IEX	2.3 for EMCV 2.0 for sindbis virus 2.0 for VSV		
DEAE IEX	1–2 for HAV	Octapharma (Vienna, Austria)	Lemon *et al.* (1994)
Ion Exchange	1–1.3 for HAV	VI technologies (Melville, NY, U.S.A.)	Hamman *et al.* (1994)
Ion exchange	4.2 for EMCV	BSD NSOB (Springe, Germany)	Mohr *et al.* (1994)
DEAE–Fractogel 650M (Factor VIII)	1.9 for BVDV 3 for HIV < 1 for PRV	CRTS/LFB (Lille, France)	Burnouf-Radosevich and Burnouf (1992)
DEAE–Fractogel 650M (vWF)	3.9 for HIV 5.0 for PRV 1.8 for VSV 1.5 for SV-40		
Gel filtration	2–3.6 for HAV	Bayer (West Haven, CT, U.S.A.)	Mitra *et al.* (1994)
Gel filtration	< 1.0 for IBR, BVDV, CPV	HemaSure A/S (Gentofte, Denmark)	Kaersgaard (1996)
Q Sepharose	2.2 for polio virus 3.0 for VSV 2.9 for BHV 0 for CPV		
S Sepharose	2.4 for polio virus 0.9 for VSV 1.6 for murine PV 1.6 for BHV 2.4 for BVDV 1.7 for MuLV		

Table 2.4. Virus partitioning over chromatographic steps in Factor IX manufacturing processes

Chromatographic step	Log reduction ($Log_{10}TCID_{50}$)	Company	Reference
DEAE Sepharose	2.1 for HIV 3.0 for VSV 3.1 for sindbis virus 1.4 for CPV	Pharmacia & Upjohn (Stockholm, Sweden)	Jernberg *et al.* (1996) Löf *et al.* (1993)
Heparin Sepharose	0.4 for HIV 0.4 for VSV 0.7 for sindbis virus 1.7 for CPV		
Cation exchange	1.7 for HIV 1.5 for VSV 2.0 for sindbis virus 0.2 for CPV		
DEAE Spherodex	> 2 for HBsAg 1.5 for HIV 1.9 for reo virus	Pasteur Mérieux Serums Vaccines (Marcy-L'Etoile, France)	Lutsch *et al.* (1993)
Monoclonal antibody affinity – Sepharose	2.0 for HBsAg > 4.5 for HIV 3.4 for reo virus		
DEAE Sepharose FF	0.8 for HBsAg >2.1 for HIV 0 for reo virus		
DEAE cellulose	2.0 for HAV	CSL Limited (Melbourne, Australia)	Johnston *et al.* (2000a)
Anion exchange	0.5 for HAV		
Heparin affinity	1.6 for HAV 1.6 for BVDV 1.9 for EMCV		
DEAE Sepharose CL6B	3.1 for HIV 2.8 for PRV 1.9 for VSV 2.2 for SV-40		Michalski *et al.* (1993)
Heparin Sepharose CL6B	2.0 for HIV 5.5 for PRV 1.9 for VSV 2.5 for SV-40		
DEAE IEX	1.4 for sindbis virus	Alpha Therapeutic Corp. (Los Angeles, CA, U.S.A.)	Herring *et al.* (1993)
Barium citrate affinity	2.0 for vaccinia virus		
Copper chelate affinity	> 5.1 for BPV 5.0 for HAV	BPL (Elstree, Hertfordshire, U.K.)	Roberts (1996b)
Copper chelate affinity	6.9 for sindbis virus ≥ 5.9 for vaccinia virus 4.6 for polio virus	BPL	Roberts (1995)
Q Sepharose	6.1 for MuLV 5.4 for BPV 4.4 for HSV 5.5 for reo virus	Genetics Institute Inc (Cambridge, MA, U.S.A.)	Adamson *et al.* (1998)
Copper chelate affinity	2.2 for BPV 3.9 for HSV 0.2 for reo virus		
Ion exchange	6.7 for HIV 5.2 for HCV model 4.2 for PRV	Immuno/ Baxter (Glendale, CA, U.S.A.)	Barrett *et al.* (1994)

(1995, 1996b) appears to provide, unequivocally, an efficient clearance (> 4 log removal) of all the viruses studied (sindbis virus, vaccinia virus, HAV, bovine parvovirus, polio virus). Using the same step, but different starting material, the results of Adamson *et al.* (1998) were not so definitive, with only herpes simplex virus (HSV) giving a high log reduction of 3.9.

Jernberg *et al.* (1996) showed no significant reduction (< 1 log) in the enveloped viruses over their heparin affinity step. In a similar process, with an affinity step following an ion exchange step, Michalski *et al.* (1993) showed 2–3 log of enveloped viruses. Both Johnston *et al.* (2000a) and Herring *et al.* (1993) obtained a consistent log reduction of 1.6–1.9 for non-enveloped viruses.

Results from ion exchange chromatography generally appear to vary from 1–3 log reduction for both enveloped and non-enveloped viruses, with the exception of the results of Barrett *et al.* (1994) who reported a log reduction of > 4 for all three enveloped viruses.

Overall reductions in combining chromatographic steps in Factor IX processes are more promising. Results of Jernberg *et al.* (1996) show that combining two ion exchange steps and an affinity step gave overall log reductions of 3.7 for HIV, 4.5 for VSV, 5.2 for sindbis virus and 3.1 for CPV. In a similar process with two ion exchange steps and an affinity step, Johnston *et al.* (2000a) showed an overall reduction of 3.6 for HIV. In the two steps studied by Michalski *et al.* (1993), overall log reduction factors were 5.1 for HIV, 8.3 for PRV, 4.7 for SV-40 virus, and 3.8 for VSV.

ALBUMIN

Of the studies collated in *Table 2.5*, there is notably a lack of congruity in the results. However, there are a few things of note. Clearance of polio virus over anion exchange steps (DEAE Sepharose and DEAE Sephadex) is limited (< 2 logs). Clearances of model viruses for HBV are also limited (< 1.0 log), with the exception of the study reported by Berglöf *et al.* (1992). Studies with HIV generally yield a log reduction factor of 3 logs. One result of Adcock *et al.* (1998a) demonstrated a 5.3 log reduction for HAV. In studies on the cation exchange steps (using CM Sepharose and COOH Sephadex), clearances of viruses are generally low, with the exception of the study of Berglöf *et al.* (1992).

There is only the one study on gel filtration, which is that used at CSL Limited. Only the HAV result of Adcock *et al.* (1998a) showed an effective log reduction factor (> 4 logs), whilst 1.5 was obtained for BVDV, and no significant reduction was observed for HBsAg (Adcock, 1998). These results were unexpected given that HAV is the smallest of the three viruses and should, therefore, have resulted in the least reduction of the three if size was the dominating factor. Thus, surface interactions must be coming into play to cause more HAV to bind to the resin than either HBsAg or BVDV.

In combining the steps from each study, some significant reduction can be claimed. Adcock *et al.* (1998a) showed a significant reduction of > 11.3 for HAV over the three chromatographic steps of CSL Limited albumin process. Not such significant reductions were observed by Cameron *et al.* (1997) for the other non-enveloped viruses, polio virus and CPV (< 4 logs in both cases). For the enveloped virus HCV and its model virus BVDV, reductions of 5.5 and 2.8 were observed by Adcock *et al.* (1998b)

Table 2.5. Virus partitioning over chromatographic steps in albumin manufacturing processes

Chromatographic step	Log reduction ($Log_{10}TCID_{50}$)	Company	Reference
DEAE Sepharose FF	5.3 for HAV 0.3 for HBsAg	CSL Limited	Adcock *et al.* (1998a)
CM Sepharose FF	1.5 for HAV 0.3 for HBsAg		Adcock *et al.* (1998b)
Sephacryl S200 HR	> 4.5 for HAV 1.5 for HBsAg		
DEAE Sepharose FF	1.6 for polio virus 0.3 for CPV	CSL Limited	Cameron *et al.* (1997)
CM Sepharose FF	1.6 for polio virus > 2.1 for CPV		
DEAE Spherodex	3.5 for HIV 1.4 for HBV 0 for polio virus	Pasteur Mérieux Serum et Vaccins	Véron *et al.* (1993)
QMA Spherosil	4.2 for HIV 1.1 for HBV 0.0 for polio virus		
DEAE Spherodex	< 1 for HBV 1.8 for polio virus > 3.3 for HIV	CRTS/LFB (Les Ulis, France)	Stoltz *et al.* (1993)
COOH Spherodex	< 1 for HBV 1.1 for polio virus > 1.2 for HIV		
QMA Spherosil,	< 1 for HBV 0.6 for polio virus > 3.5 for HIV		
DEAE Sepharose FF	1.3 for BVDV	CSL Limited	Adcock *et al.* (1998b)
CM Sepharose FF	0.4 for BVDV		
Sephacryl S200 HR	1.5 for BVDV		
DEAE Sepharose FF	5.0 for BHV	Pharmacia Biotech (Uppsala, Sweden)	Berglöf *et al.* (1992)
CM Sepharose FF	7.1 for BHV		
DEAE Sepharose FF CM Sepharose FF	3.5–6.4 for BHV < 1.5 for BVDV > 7 for EMC > 5.2 for HIV	Pharmacia Biotech	Andersson *et al.* (1996)

and Johnston *et al.* (2000b), respectively. Using identical resins and subtly different operating conditions, Berglöf *et al.* (1992) and Andersson *et al.* (1996) showed significant log reduction factors for BHV (12.1 logs), HIV (> 5.2 logs), and EMCV (> 7 logs). Using different resins and subtly different operating conditions, Stoltz *et al.* (1993) reported a low log reduction factor for polio virus (2.9 logs), yet a significant reduction for HIV (> 8.0 logs) over three ion exchange steps. Véron *et al.* (1993) at the Pasteur Mérieux Serum and Vaccins (Marcy-L'Etoile, France) and Stoltz *et al.* (1993) at LFB reported 7.7 and 8.0 log reduction factors for HIV, respectively, over the ion exchange processes.

IMMUNOGLOBULIN IgG

There are fewer published studies on chromatographic steps in the manufacturing processes of IgG as today these products are predominantly made by a cold ethanol process, coupled with a VI step (s). Those studies reported in the literature are generally favourable for reducing virus load during several chromatographic steps. Johnston *et al.* (2000b) showed > 5.3 log reduction of BVDV over two ion exchange steps, Andersson *et al.* (1996) showed > 4 log reduction factor for BHV, EMCV and HIV over three ion exchange steps, and Burnouf (1993) demonstrated > 4 log reduction factor for HIV, PRV, sindbis virus and PPV over two ion exchange steps. Kaersgaard (1996) and Bees *et al.* (1989) showed > 4 log reduction factors for all virus studies. These results demonstrate that chromatography plays a significant role in decreasing the risk of viral transmission in IgG products.

OTHER PRODUCTS

There are even fewer studies looking at the small volume products, such as antithrombin III, α1PI, thrombin and Factor VII. These products are included in *Table 2.7*. Briefly, Burnouf (1993), Biescas *et al.* (1998), and Johnston *et al.* (2000a) demonstrated some reduction of a range of viruses (HIV, SV-40, VSV, PRV, BVDV and HAV) over a heparin affinity step in the manufacturing of antithrombin III. Finally, in purifying Factor VII from a recombinant source, Kaersgaard (1996) showed that a monoclonal antibody affinity chromatography gave considerable reduction of three enveloped viruses, SV-40, PRV and reo virus.

Discussion

The variability of the data observed in *Tables 2.3* to *2.7* highlights the complex nature of virus partitioning on chromatographic resins. Variables such as virus size, symmetry, membrane structure, surface topography and surface charge, in addition to operating conditions such as buffer pH, ionic strength, flow rate, resin porosity and ligand density all play some role in the interactions between virus-protein-ligand-resin. Knowing the isoelectric point (pI) of a virus does not seem to be predictive of its behaviour on an ion exchange resin. Cameron *et al.* (1997), for example, demonstrated notably different clearance patterns of polio virus and CPV, viruses with similar pIs. Knowing the size of the virus does not help predict its performance on a size exclusion, gel filtration column. Adcock (1998) demonstrated that, in fact, the

Table 2.6. Virus partitioning over chromatographic steps in IgG manufacturing processes

Chromatographic step	Log reduction ($Log_{10}TCID_{50}$)	Company	Reference
DEAE Sepharose FF	1.4 for BVDV 1.2 for HCV 1.7 for HAV	CSL Limited	Johnston *et al.* (2000b)
Macroprep HQ	> 3.9 for BVDV		Adcock *et al.* (1998a)
DEAE Sepharose FF Q Sepharose FF CM Sepharose FF	3.4–6.4 for BHV < 2.4 for BVDV > 6 for EMCV > 5.6 for HIV	Pharmacia Biotech	Andersson *et al.* (1996)
2 × IEX	> 8.6 for HIV 7.6 for PRV 4.0 for sindbis virus 10.6 for PPV	LFB	Burnouf (1993)
DEAE cellulose	> 8.5 for VSV > 6.0 for SV-40 > 4.8 for BVDV	HemaSure A/S	Kaersgaard (1996)
DEAE Spherosil W 1000	> 4.2 for HBsAg	Pasteur Institute Mérieux	Tayot *et al.* (1987)
DEAE Sephadex A50	4.0 for HIV 0 for sindbis virus 0.4 for IBR	Winnipeg Rh Institute (Winnipeg, Canada)	Bees *et al.* (1989)
DEAE Sepharose	5.0 for HIV 2.9 for sindbis virus 2.4 for IBR		
DEAE Biogel	7.2 for HIV 1.7 for sindbis virus 1.7 for IBR		

Table 2.7. Virus partitioning over chromatographic steps in the manufacturing processes of other plasma proteins

Product	Chromatographic step	Log reduction ($Log_{10}TCID_{50}$)	Company	Reference
ATIII	Heparin Sepharose CL6B	1.6 for HIV 1.0 for SV-40 2.4 for VSV 4.0 for PRV	LFB (formerly CRTS)	Burnouf (1993)
ATIII	Heparin Agarose	3.8 for HIV < 1.0 for BHV 2.9 for BVDV < 1.0 for EMCV	Institute Grifols	Biescas *et al.* (1998)
ATIII	Heparin Sepharose CL6B	2.5 for BVDV 1.5 for HAV	CSL Limited	Johnston *et al.* (2000b)
FVII	Monoclonal antibody affinity	2.8 for SV-40 2.3 for VSV 5.1 for reo virus	HemaSure A/S	Kaersgaard, (1996)
α1PI	Sephacryl S200 HR	> 4.5 for HIV 1.5 for Thieler virus	LFB (Formerly CRTS)	Burnouf (1995)

smallest virus, HAV, gave significantly greater clearance than the larger viruses, BVDV and HBsAg. What do we know about viruses then that can help predict chromatographic interactions?

We know that electrostatic forces steer the protein towards the charged resin (Sadana, 1992). We know that viruses are generally negatively charged at neutral pH, and it has been frequently noted viruses will bind to some filter membranes as a result of electrostatic attraction (Brock, 1983). Downing *et al.* (1992) and Noinville *et al.* (1995) speculate on the electrostatic interactions, including van der Waals forces and non-polar interaction of viruses with columns. Shields and Farrah (1983) indicate that hydrophobic interactions play some role in the adsorption of viruses to filter membranes and that disruption of the interaction by the addition of salts and alcohol or detergents permitted efficient elution of polio virus from filter membranes. We know that it is the outer surface properties of viruses that define their behaviour on surfaces. Viruses with an outer membrane containing an even distribution of positively and negatively charged proteins, and hence an even distribution surface charge, may have virus-column interactions governed by their net charge (apparent charge). On the other hand, viruses with outer membranes which contain viral protein sub-units clustered about particular local axes of symmetry may localize the charge density of the virus, thus influencing binding and partitioning, regardless of the apparent net charge (Burnouf, 1993). Complexing of blood-borne viruses by neutralizing antibodies present in the feed samples may change the surface characteristic of the virion, whether it be its overall surface charge, charge density or hydrophobicity, thus influencing the behaviour of the viruses during ion-exchange chromatography. Recent data presented by Adcock *et al.* (1999) showed significantly greater reduction of HAV in the absence of antibodies (> 5.1 logs) than in the presence of antibodies (1.4 logs). As HBV and HCV are lipid-enveloped viruses, with outer membrane projections comprising of glycoproteins, effective binding to a chromatographic support may be conferred by hydrophobic interactions with the lipid membrane and/or by ionic interactions with glycoprotein projection.

The selection of relevant and model viruses used during these studies also remains critical to the validity of the clearance data obtained. Model viruses may partition in a completely different manner to the target virus because of relatively minor differences in surface properties, such as glycosylation. Even a relevant virus propagated in the laboratory (ie HAV) may act differently from wild-type virus in this respect. This deficiency in the selection of a model virus was highlighted during anion-exchange chromatography partitioning studies conducted with HAV by Adcock *et al.* (1998a) and the model virus, polio virus, by Cameron *et al.* (1997), where significant reduction was seen with HAV but not with polio virus. Other conflicting examples are illustrated by Bees *et al.* (1989) and Burnouf (1993). On the other hand, several studies showed comparable results between sindbis virus and HIV (Jernberg *et al.*, 1996; Schreiber *et al.*, 1989) between EMCV and HAV (Johnston *et al.*, 2000a) and between BVDV and HCV (Johnston *et al.*, 2000b).

The underlying message from these studies is that accurate data must be obtained for each individual chromatographic step for each virus, where possible using the relevant virus, to ensure an accurate and realistic risk assessment. However, clearance data generated from different model viruses may be useful in indicating the potential of a chromatographic process/matrix to reduce the viral load of 'unknown viruses'.

The area of research investigating the parameters governing virus-matrix interaction will need to expand to keep pace with the growing popularity of chromatography. As future advances in the field of matrix chemistry and design can be expected to lead to the development of new chromatographic technologies, such research will assist manufacturers in selecting conditions which are likely to resolve viruses from target proteins and at the same time provide high selectivity and high yields for those target proteins.

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