Table 2. Removal of PrP^{Sc} in the preparation of high purity factor VIII (Liberate®) and fibrinogen

Process step[a]	Factor VIII		Fibrinogen	
	CF[b]	RF[c]	CF	R
1 Cryoprecipitation	1.7	1.0	1.7	1.
14 Zinc precipitation + Al $(OH)_3$ adsorption	2.0	1.7	2.0	1.
15 SD[d] + DEAE Tyopearl 650 M chromatography	3.8	3.1	≥4.1	≥
16 Membrane filtration (0.45 μm/0.22 μm)	1.6	1.0	n/d[e]	n/

a Number of process step in flowsheet (fig. 1).
b PrP^{Sc} clearance factor (log_{10}).
c PrP^{Sc} reduction factor (log_{10}).
d Solvent-detergent treatment.
e Not determined.

Table 3. Removal of PrP^{Sc} in the preparation of high purity factor IX concentrate (HIPFIX®), factor IX complex (HTDEFIX®) and thrombin

Process step[a]	Factor IX		FII, IX and X		Thrombin	
	CF[b]	RF[c]	CF	RF	CF	RF
1 Cryoprecipitation	<1.0	<1.0	<1.0	<1.0	<1.0	<
2 DEAE-cellulose adsorption	2.8	3.0	2.8	3.0	2.8	3.(
11 DEAE-sepharose chromatography	4.4	3.0	n/a[d]	n/a	n/a	n/a
12 SD[e] + heparin-sepharose chromatography	2.7	1.4	n/a	n/a	n/a	n/a
16 SD + S-sepharose chromatography	n/a	n/a	n/a	n/a	3.3	2.9

a Number of process step in flowsheet (fig. 1).
b PrP^{Sc} clearance factor (log_{10}).
c PrP^{Sc} reduction factor (log_{10}).
d Not applicable.
e Solvent-detergent treatment.

Table 4. Distribution of PrP^{Sc} by precipitation

Process step[a]	Precipitation conditions				% distribution[b] of PrP	
	ethanol %	pH	temperature °C	time h	precipitate	supernata
1 Cryoprecipitation	–	–	–	–	10	96
3 FrI+II+III precipitation	21	6.70	–5.0	15	84.4	4.7
4 FrIV precipitation	35	5.55	–5.0	17	>100	<0.1
8[c] FrI+III precipitation	8	5.10	–2.5	16	>100	<0.02
8[d] FrI+III precipitation	12	5.10	–2.5	16	>100	<0.02

a Number of process step in flowsheet (fig. 1).
b 100% = Total PrP^{Sc} measured in feedstock prior to precipitation.
c Process step used in the preparation of immunoglobulins for intramuscular administratio
d Process step used in the preparation of immunoglobulins for intravenous administration.

ly a small proportion of PrP^{Sc} could be accounted for in samples taken over chromatographic procedures, e.g., about 0.1% at steps 2, 11 and 13 (table 3). It is possible that PrP^{Sc} may have partitioned into wash fractions which were not sampled; however, it seems more probable, given its adherent nature [16], that most PrP^{Sc} remained adsorbed to chro matographic matrices following product elution.

The contribution made by each step in an overall proces will be dependent on whether or not different steps are com plementary to one another. As each process step was exam

Partitioning over cryoprecipitation is less clear. On processing plasma from mice experimentally infected with a human TSE, the infectivity appeared to partition primarily into the cryoprecipitate, whilst in the comparative exogenous experiment using human blood 'spiked' with scrapie 263K, 8.1 $\log_{10}$ LD_{50} remained in plasma, but only 0.7% of this infectivity was detected in the cryoprecipitate [30]. In a subsequent larger-volume endogenous experiment, using blood from scrapie-infected hamsters, Rohwer [38] has estimated that about 20% of the plasma infectivity partitioned into cryoprecipitate. By contrast Petteway et al. [31], using human plasma to which scrapie 263K brain homogenate was added, reported that 90% of PrP^{Sc} partitioned into the cryoprecipitate. Our finding that about 10% of the scrapie 263K PrP^{Sc} added to human plasma partitioned into cryoprecipitate (table 4) is reasonably comparable with Rohwer's [38] figure of 20% from his larger-volume endogenous model, suggesting that the microsomal inoculum used in our study behaved similarly to a TSE agent present naturally in plasma. However, it is also possible that some of the different results reported may simply reflect variations between different manufacturer's procedures for the preparation of cryoprecipitate, rather than differences in the nature of the infective materials used.

Little information is available on the behaviour of TSE agents in chromatographic separations currently used in plasma fractionation. Drohan [34], in a study of factor VIII processing, has reported $\log_{10}$ RFs of 4.4 and 6.3 for immunoaffinity and ion exchange chromatography, respectively, using a 10% brain homogenate of hamster-adapted scrapie as the inoculum and with infectivity determined by bioassay. Additional chromatographic data are available from a variety of different bio-process industries [13, 14, 16, 39, 40] with $\log_{10}$ RFs ranging from 2.2 to 5.5. Our results on ion exchange are within this range, with essentially no difference being observed between anion exchange and cation exchange or between different ion exchange matrices (table 3). The somewhat smaller degree of PrP^{Sc} reduction observed over heparin-affinity chromatography (table 3; step 12) may have been due to a smaller charge difference, together with the relatively high concentration of sodi chloride (i.e. 500 m*M*) at product elution. The probabi that most PrP^{Sc} remained bound to chromatographic ma ces emphasises the importance of either limiting the re- of adsorbents or in developing suitable cleaning procedu

The observation that a high degree of PrP^{Sc} removal be obtained by depth filtration (table 1) may be the most i portant finding from our study, as depth filters are stand items used in most albumin and immunoglobulin process In addition, the low cost of filter media means that fil pads are normally disposed of after each use, thereby avo ing the possibility that subsequent product batches could exposed to any TSE infectivity which might have been sorbed.

Conclusions

The data obtained from our study suggest that if vC infectivity was present in plasma pools obtained from U donors, then the quantity of abnormal prion present wo have been reduced substantially during the preparation each of the plasma products manufactured by SNB Whether or not these processes would have been capable completely removing all vCJD infectivity is not known, this may have been dependent on the quantity of vCJD fectivity actually present as well as on the accuracy of experimental models from which our data were obtain Further studies are required to address these points.

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Assessment of the potential of plasma fractionation processes to remove causative agents of transmissible spongiform encephalopathy

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SUMMARY. Although there is no evidence that classical CJD (cCJD) can be transmitted by human blood or blood products in clinical practice, uncertainties surrounding new variant CJD (nvCJD) have led to the safety of plasma products derived from UK donors being questioned. To better define whether or not there is a risk of nvCJD being transmitted it is necessary to determine how the causative agent would partition across the separations processes used in the preparation of plasma products.

The abnormal prion protein which is associated with transmissible spongiform encephalopathies (TSEs), such as CJD, has a low solubility, a high tendency to form aggregates and adheres to surfaces readily. If the physicochemical properties of the agent of nvCJD are similar to those of abnormal prion protein then nvCJD may be removed by precipitation and adsorption technologies used in plasma fractionation.

Available data on the removal of TSE agents by such bioprocess technologies have been used to estimate the potential degree of reduction expected from each step in the plasma fractionation processes used by the SNBTS. The overall process reduction factors estimated are: 10^{13} (albumin), 10^9 (immunoglobulins), 10^7 (factor IX, thrombin), 10^5 (fibrinogen), 10^4 (factor VIII) and 10^3 (factor II, IX and X); however, it will be necessary to establish the accuracy of these estimates by practical validation studies.

Key words: Creutzfeldt–Jakob disease, new variant CJD, partitioning of CJD/scrapie, plasma fractionation, plasma products.

Transmissible spongiform encephalopathies (TSEs) are a group of fatal neurodegenerative disorders including scrapie in sheep, bovine spongiform encephalopathy (BSE) in cattle and Creutzfeldt–Jakob disease (CJD) in humans (Baker & Ridley, 1996). CJD is a rare disease which occurs uniformly world-wide, with an incidence of about 1 per 10^6 persons per annum. A new form of TSE in humans, termed new variant CJD (nvCJD), was first identified in 1996 in the UK and is believed to have resulted from the consumption of central nervous tissue from BSE-infected animals which entered the human food chain (Will *et al.*, 1996). The current clinical incidence of nvCJD in the UK is about 0·2 per 10^6 persons per annum (Scottish Centre for Infection & Environmental Health, 1998) but, in the absence of a suitable diagnostic procedure, the subclinical prevalence of the infection is not known.

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Transmission of CJD by human growth hormone derived from human pituitary glands and by other medical procedures (Brown *et al.*, 1992) has led to concerns that BSE could be transmitted by medicinal products manufactured using bovine substances (CPMP, 1992) and that CJD may be transmissible by products derived from infected blood donors (Esmonde *et al.*, 1993).

A number of studies have been undertaken to determine whether or not CJD has been transmitted by blood or plasma products, with no evidence of transmission being found to date (Brown, 1995; Ricketts *et al.*, 1997; Evatt *et al.*, 1998; Evatt, 1998). This apparent absence of CJD transmission could be due to the disease not being transmissible by blood or blood products in clinical practice, the low prevalence of the disease in the blood donor population, the absence or low concentration of the CJD agent in plasma for fractionation, the removal of the causative agent of CJD by the processes used to manufacture human plasma products or to a long incubation period for the disease in recipients.

Because of uncertainty over the safety of plasma

products in this regard, the FDA decided that batches of plasma products must be recalled where a donor had been diagnosed with CJD or was at increased risk of CJD (FDA, 1995). In the 12 months to 30 March 1998, the FDA recalled 175 batches of albumin products, 83 batches of immunoglobulins and 11 batches of coagulation factor concentrates on this basis. This extent of plasma product recall in North America resulted in shortages of critical therapeutic products (FDA, 1998a). Subsequently, the FDA position was revised to recommend the recall of products only where a donor had developed nvCJD (FDA, 1998b). In Europe, plasma products do not require to be recalled on the basis of classical CJD (cCJD), but a decision was taken to recall batches where nvCJD has been diagnosed in a contributing donor (CPMP, 1998). Three such UK donors were identified in 1997 and the subsequent product recalls, the lack of knowledge of the prevalence of subclinical nvCJD in the UK population together with some evidence that the distribution of nvCJD in human tissues may differ from that of cCJD (Hill *et al.*, 1997) resulted in the safety of plasma products derived from UK donors being questioned (Ludlam, 1997) and ultimately to a decision by the UK Government to ban the manufacture of plasma derivatives from plasma collected in the UK, as a precautionary measure (Warden, 1998).

In order to define the risk of either cCJD or nvCJD being transmitted by plasma products it is necessary to determine how the causative agents would partition across the separations processes that are employed in the manufacture of plasma products. The effect of pharmaceutical manufacturing procedures on TSE agents is normally assessed by challenging a scaled-down version of the process with a high titre of a defined strain of a rodent adapted scrapie agent and measuring the infectivity of samples, taken before and after processing, by intracerebral injection in animals. Such studies take a long time to complete and, because of the high costs involved, tend to be restricted to a small number of key process steps rather than a comprehensive examination of the complete manufacturing process. For example, in a study of the process used to manufacture Trasylol®, the examination of four individual process steps consumed 1600 mice and took 3 years to complete (Kozak *et al.*, 1996).

TSE agents are highly resistant to inactivation (Taylor, 1996) and therefore, for protein pharmaceuticals, it is their physical removal that is of particular interest. Preliminary data on TSE agent partitioning have been reported for some selected process steps used in the fractionation of human plasma using a rodent adapted strain of a human TSE agent (Brown *et al.*, 1998) and a rodent adapted strain of the scrapie agent (Brown *et al.*, 1998; Petteway *et al.*, 1998), but the outcomes expected over a complete plasma fractionation process have : yet been described. In the absence of comprehens measurements of TSE agent partitioning across plas fractionation processes, the behaviour of nvCJD can estimated only by extrapolation of data obtained fr similar biopharmaceutical process operations. A pro sional assessment of how TSE agents might be expec to partition during plasma fractionation has been ma on this basis.

PLASMA FRACTIONATION

The Scottish National Blood Transfusion Servi (SNBTS) manufactures over 250 000 unit doses of range of different plasma products from ≈100 000 kg plasma. The preparation of each product involves exte sive processing via a carefully designed, closely cc trolled series of operations (Fig. 1) (Foster, 1994). Ea process includes a number of steps in which mac molecular constituents are preferentially removed; the steps are summarized below on a product-by-produ basis.

Albumin (Alba®)

The SNBTS process for the manufacture of album involves removal by centrifugation of the precipita which forms when the frozen donations of plasma a thawed (cryoprecipitate), removal by centrifugation the precipitates which form at 21% ethanol, pH 6·7 , −5 °C (fraction I + II + III) and at 35% ethanol, pH 5·5 −5 °C (fraction IV), depth filtration through a mix bed of cellulose, kieselguhr and perlite at two stag depth filtration through a mixed bed filter incorporati a cation exchange resin and membrane filtration three different stages of the process, two of whi employ a cellulose acetate membrane. The final produ is pasteurized at 60 °C for 10 h to inactivate potenti viral contaminants.

Immunoglobulins

Similar purification procedures are used in the man facture of immunoglobulin products. Following tl removal of cryoprecipitate and the recovery of fractic I + II + III, the resuspended fraction I + II + III adjusted (8 or 12% ethanol, pH 5·1, −3 °C) to precipita fraction I + III, which is removed by centrifugation, tl supernatant being clarified by borosilicate glass dep filtration. The IgG solution is subsequently subjected to mixed bed depth filtration (cellulose, kieselguhr ar perlite) and to membrane filtration at three differe stages of manufacture, two of which employ a cellulo acetate or similar membrane.

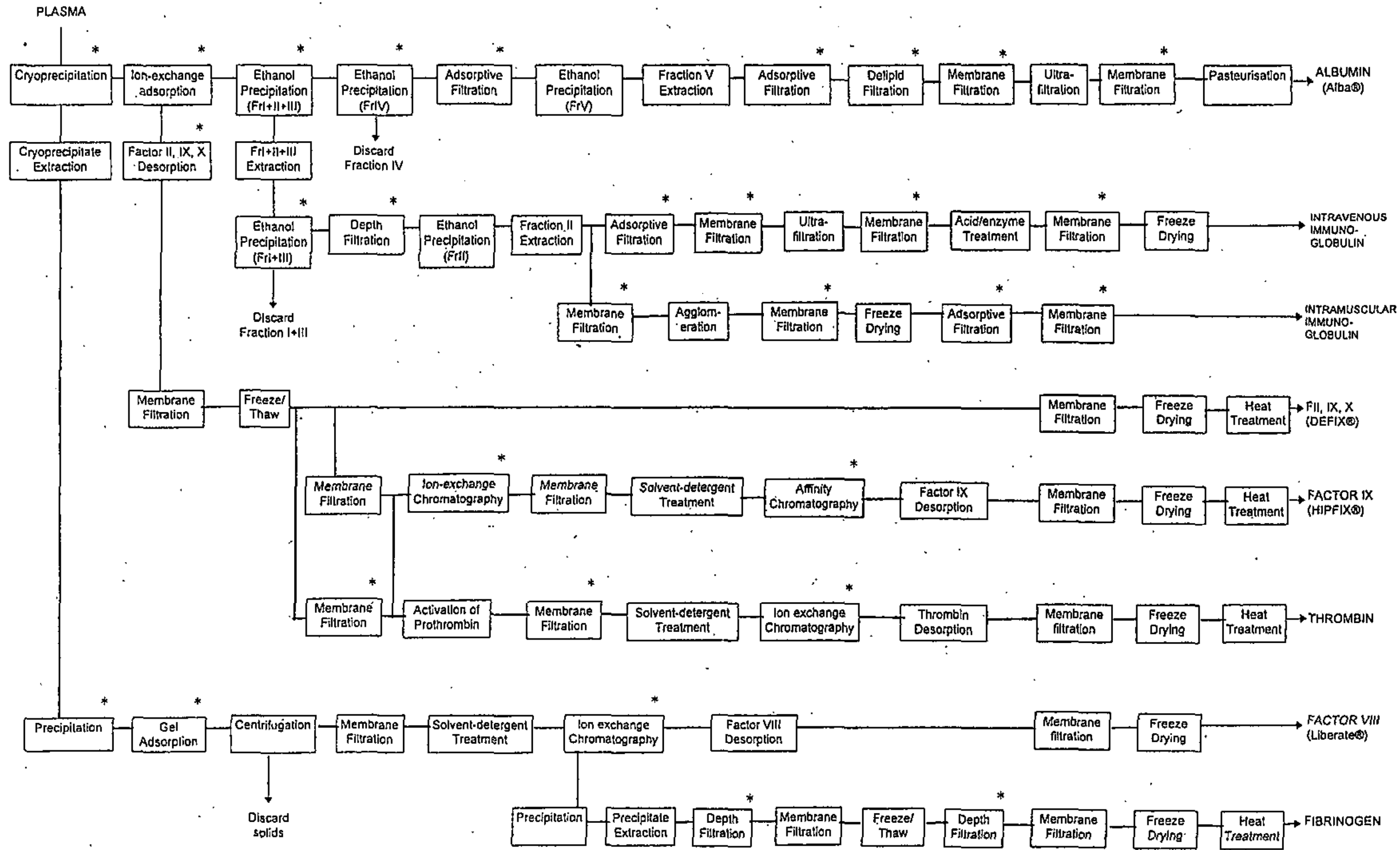

Fig. 1. SNBTS process for the fractionation of human plasma. *Denotes step estimated to have potential for TSE removal or reduction.

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Factor II, IX and X concentrate (DEFIX®)

The supernatant which remains following the removal of cryoprecipitate is subjected to a batch anion exchange adsorption, with coagulation factors II, IX and X being recovered by chromatographic desorption. The solution containing factors II, IX and X then undergoes two separate membrane filtration operations prior to being freeze dried and heat treated at 80 °C for 72 h for virus inactivation.

Factor IX concentrate (HIPFIX®)

Factor IX concentrate is purified from the desorbed factor II, IX and X eluate (above) using anion exchange chromatography and heparin affinity chromatography. A total of five separate membrane filtration steps are employed, as well as a solvent-detergent treatment to inactivate lipid-enveloped viruses, prior to the product being freeze dried and heat treated at 80 °C for 72 h.

Thrombin

Thrombin is also purified from the desorbed factor II, IX and X solution, in this instance by cation exchange chromatography, with a total of six separate membrane filtration steps (two of which employ a cellulose acetate membrane) and a solvent-detergent treatment prior to the product being freeze dried and heat treated at 80 °C for 72 h. Albumin (Alba®) is added as a stabilizer and must also be considered in the assessment of risk.

Factor VIII concentrate (Liberate®)

In the preparation of factor VIII concentrate, the extract obtained from cryoprecipitate is partially purified by precipitation and by adsorption with aluminium hydroxide gel. Following removal of the solids by centrifugation, the supernatant is treated with tri(*n*)-butyl phosphate + polysorbate 80 for the inactivation of lipid-enveloped viruses and by anion exchange chromatography for further purification of factor VIII. Membrane filtration is employed at two different stages of processing.

Fibrinogen

The preparation of fibrinogen is similar to that of factor VIII except that the unadsorbed fraction from anion exchange chromatography is processed rather than the desorbed fraction. The fibrinogen-rich solution is then subjected to three precipitation operations followed by two depth filtration and three membrane filtration procedures prior to freeze drying and heat treatment at 80 °C for 72 h.

THE PARTITIONING OF TSE AGENTS IN BIO-SEPARATION PROCESSES

Background

Although a number of different TSE diseases are know the causative agents are generally believed to posse similar physicochemical properties (Groschup *et a* 1997) and to consist of a conformationally altered for of cellular prion protein (PrP^{c}), referred to as abnorm prion protein (e.g. PrP^{Sc}). Whether or not PrP^{Sc} is itse the causative agent of disease is not known; howeve removal of PrP^{Sc} is generally associated with removal infectivity (Farquhar *et al.*, 1998).

PrP^{Sc} has still to be fully characterized (Donne *et a* 1997; Edenhofer *et al.*, 1997), but the molecule believed to be based on a 27–30-kDa glycoprote subunit (Meyer *et al.*, 1986) and, with both hydrophob and hydrophilic domains (Bolton *et al.*, 1987), tends form large amorphous or rod-shaped aggregates *in vit* (McKinley *et al.*, 1991). PrP^{Sc} has a low aqueous solubili below pH 9 (Gasset *et al.*, 1993) and is readily precipitate by ethanol (Prusiner *et al.*, 1980), ammonium sulpha and polyethylene glycol (PEG) (Turk *et al.*, 1988).

Therefore, it can be postulated that certain biosepar tion technologies that are used in the preparation plasma products, such as precipitation, adsorption an filtration, may well be capable of removing significa quantities of the abnormal prion protein associated wi nvCJD. Indeed, the potential of these technologies f the removal of TSE agents has been identified previousl in guidelines concerning the preparation of medicin products (CPMP, 1992).

Measurement of TSE agent partitioning

Most information on the partitioning of TSE agents ha been obtained from studies in which the behaviour rodent adapted scrapie agent (PrP^{Sc}) was measure PrP^{Sc} has similar biochemical properties to cCJD (Benc heim *et al.*, 1985) and has been accepted by Regulator Authorities as a suitable model for studies of the inacti vation and removal of BSE (Bader *et al.*, 1998). nvCJ is believed to be the human form of BSE (Almond & Pattison, 1997) and therefore PrP^{Sc} is also likely to b regarded as a suitable marker for determining the parti tioning behaviour of the agent of nvCJD. Nevertheless, is by no means sure that data from animal model system are predictive for the human situation.

The transmissibility or infectivity of a TSE agent ma be influenced by the strain of agent used, the dose of th agent, the route of administration and the presence o absence of a species barrier. Most studies of the infec tivity of TSEs measure the dose that causes infection i 50% of the animals tested (ID_{50}), following inoculatio

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