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Studies on the Removal of Abnormal Prion Protein by Processes Used in the Manufacture of Human Plasma Products

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Abstract

Background and Objectives: To identify if any process steps used in plasma fractionation may have a capability of removing agents of human transmissible spongiform encephalopathy (TSE). ***Materials and Methods:*** Sixteen fractionation steps were investigated separately by adding a preparation of hamster adapted scrapie 263K to the starting material at each process step and determining the distribution into resultant fractions of protease-K-resistant (abnormal) prion protein by Western blot analysis. ***Results:*** A number of process operations were found to remove abnormal prion protein to the limit of detection of the assay. These were cold ethanol precipitation of fraction IV (log reduction, LR, ≥3.0) and a depth filtration (LR ≥ 4.9) in the albumin process; cold ethanol fraction I+III precipitation (LR ≥3.7) and a depth filtration (LR ≥2.8) in the immunoglobulin processes and adsorption with DEAE-Toyopearl 650M ion exchanger (LR ≥ 3.5) in the fibrinogen process. In addition, a substantial degree of removal of abnormal prion protein was observed across DEAE-Toyopearl 650M ion exchange (LR = 3.1) used in the preparation of factor-VIII concentrate; DEAE-cellulose ion exchange (LR = 3.0) and DEAE-sepharose ion exchange (LR = 3.0) used in the preparation of factor-IX concentrates and S-sepharose ion exchange (LR = 2.9) used in the preparation of thrombin. ***Conclusions:*** Plasma fractionation processes used in the manufacture of albumin, immunoglobulins, factor-VIII concentrate, f tor-IX concentrates, fibrinogen and thrombin all cont steps which may be capable of removing causal agents of human TSEs.

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Introduction

The fatal neurodegenerative disorder Creutzfeldt-Ja disease (CJD) has been transmitted iatrogenically vi number of routes [1] suggesting the possibility that causative agent might also be transmissible by blood pr ucts [2]. The identification of a new form of human tra missible spongiform encephalopathy (TSE), 'new varia CJD (vCJD) [3, 4], confirmation of an association with agent of bovine spongiform encephalopathy (BSE) [5 and evidence that the distribution of the agent of vCJ human tissues may differ from that of classical CJD [7 led to increased concern that vCJD may be transmissi by plasma products [9]. Consequently, as a precaution measure, the UK government decided to ban the fractio tion of plasma donated in the UK [10] and replaced with plasma purchased outside the UK. Despite this portation of plasma, the risk of transmission of vCJD mains to be defined for those patients who previously been treated with plasma products derived from donors.

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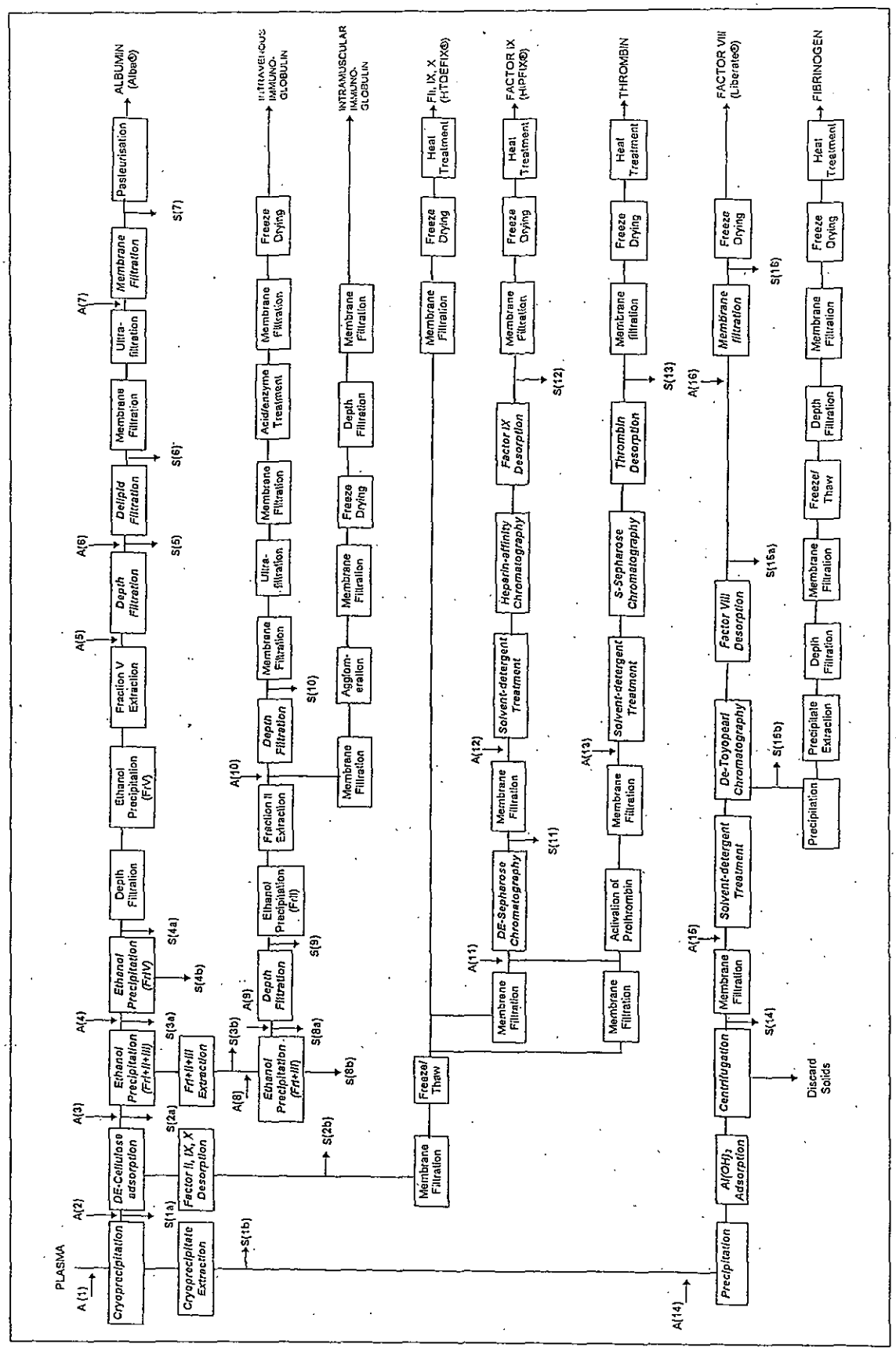
PLASMA
A(1)
Cryoprecipitation
A(2)
DE-Cellulose adsorption
A(3)
Ethanol Precipitation (Frl+II+III)
A(4)
Ethanol Precipitation (FrIV)
Depth Filtration
Ethanol Precipitation (FrV)
Fraction V Extraction
A(5)
Depth Filtration
A(6)
Delipid Filtration
Membrane Filtration
Ultra-filtration
A(7)
Membrane Filtration
Pasteurisation
ALBUMIN (Albaⓡ)
S(7)
S(6)
S(5)
S(4a)
S(4b)
S(1a)
Cryoprecipitate Extraction
S(1b)
Factor II, IX, X Desorption
S(2a)
S(2b)
Frl+II+III Extraction
S(3a)
S(3b)
A(8)
Ethanol Precipitation (Frl+II)
S(8a)
S(8b)
A(9)
Depth Filtration
S(9)
Ethanol Precipitation (FrI)
Fraction II Extraction
A(10)
Depth Filtration
Membrane Filtration
Ultra-filtration
Membrane Filtration
Acidenzyme Treatment
Membrane Filtration
Freeze Drying
S(10)
Agglom-eration
Membrane Filtration
Freeze Drying
Depth Filtration
Membrane Filtration
INTRAMUSCULAR IMMUNO-GLOBULIN
Membrane Filtration
Freeze Drying
Heat Treatment
Fll, IX, X (HTDEFIX®)
Membrane Filtration
Freeze Drying
Heat Treatment
FACTOR IX (HPFIX®)
S(12)
A(12)
Solvent-detergent Treatment
Heparin-affinity Chromatography
Factor IX Desorption
Membrane Filtration
S(11)
A(11)
DE-Sepharose Chromatography
Membrane Filtration
A(13)
Solvent-detergent Treatment
S-Sepharose Chromatography
Thrombin Desorption
Membrane filtration
Freeze Drying
Heat Treatment
THROMBIN
S(13)
Membrane Filtration
Activation of Prothrombin
Membrane Filtration
A(16)
Membrane filtration
Freeze Drying
FACTOR VIII (Liberateⓡ)
S(16)
A(15)
Solvent-detergent Treatment
Factor VIII Desorption
S(15a)
De-Toyopearl Chromatography
S(15b)
Membrane Filtration
Centrifugation
S(14)
Discard Solids
Precipitation
Depth Filtration
Precipitate Extraction
Precipitation
Membrane Filtration
Freeze/ Thaw
Depth Filtration
Membrane Filtration
Freeze Drying
Heat Treatment
FIBRINOGEN
A(14)
Al(OH)3 Adsorption
Membrane Filtration
Freeze/ Thaw

phosphate (5 m*M*) at pH 6.2. Factor II, IX and X solution (210 ml) was applied to the column, which was then treated with 150 ml wash buffer, all at a flow rate of 8.4 ml/min, followed by 10 ml of wash buffer + 280 m*M* sodium chloride at 1.9 ml/min. Factor IX was eluted using 100 ml wash buffer +360 m*M* sodium chloride, pH 7.8 at 1.9 ml/min.

Solvent-Detergent Treatment and Affinity Chromatography of Factor IX (Step 12)

Microsomal inoculum (10 ml) was added to a solution of factor IX (108 ml) which had been prepared by diluting 36 ml of factor IX eluate (step 11) with 72 ml of a solution of citrate (20 m*M*) + arginine (4.5 g/l), at pH 7.55. Tri(n-butyl)phosphate and Tween-80 were added to 108 ml of 'spiked' factor IX solution to achieve a final concentrations of 0.3 and 1%, respectively [24], the mixture stirred at 25 °C for 19 h, then purified by affinity chromatography based on the method of Burnouf et al. [25]. 30 ml heparin-sepharose FF (Pharmacia) was packed into a 26-mm diameter chromatography column (XK 26/20, Pharmacia) using 20 m*M* citrate. The solvent-detergent (SD)-treated factor IX mixture was applied to the column, the bed washed with 100 ml of 20 m*M* citrate, treated with 100 ml of 20 m*M* citrate + 200 m*M* sodium chloride and factor IX then eluted with 100 ml of a solution of citrate (20 m*M*) + arginine (4.5 g/l) + sodium chloride (500 m*M*), all at a flow rate of 3.1 ml/min.

SD Treatment and Ion Exchange Chromatography of Thrombin (Step 13)

Microsomal inoculum (9.5 ml) was added to an unpurified solution of thrombin (197 ml), which had been prepared by calcium activation of the factor II, IX and X eluate (fig. 1; step 2) according to the method of MacGregor et al. [26]. Tri(n-butyl)phosphate and Tween-80 were added to achieve final concentrations of 0.3 and 1.0%, respectively, and the mixture stirred at 25 °C for 19 h prior to purification of thrombin by ion exchange chromatography. 20 ml S-sepharose (Pharmacia) was packed into a 26-mm diameter chromatography column (XK 26/10, Pharmacia) and washed with 20 m*M* trisodium citrate (80 ml) at pH 6.5. The SD-treated thrombin mixture was applied to the column at a flow rate of 8.5 ml/min; the column was washed with 200 ml trisodium citrate (20 m*M*) and thrombin was then eluted with 80 ml of trisodium citrate (20 m*M*) + sodium chloride (500 m*M*) at a flow rate of 4.2 ml/min.

Precipitation and Adsorption of Cryoprecipitate Extract (Step 14)

Microsomal inoculum (9.5 ml) was added to cryoprecipitate extract (215 ml) which had been prepared by resuspending 48 g of frozen washed cryoprecipitate in 20 m*M* Tris (168 ml) at 20 °C. The pH of the extract was adjusted to 6.7 and zinc precipitant (zinc acetate + sodium chloride + trisodium citrate + heparin) added to obtain final concentrations of 0.5 m*M* zinc, 1 m*M* citrate and 2.5 IU/ml heparin. The mixture was stirred for 5 min at 20 °C, aluminium hydroxide (Al-hydrogel, Superfos, Copenhagen, Denmark) was added to a final concentration of 5%; after stirring for a further 15 min, the suspension was centrifuged at 5,500 *g* for 15 min at 20 °C to recover the supernatant, which was then formulated to 20 m*M* trisodium citrate and 2.5 m*M* calcium chloride.

SD Treatment and Ion Exchange Chromatography for Factor VIII and Fibrinogen (Step 15)

Microsomal inoculum (10 ml) was added to a solution of fact VIII (104 ml) of intermediate purity, containing 20 m*M* trisodium c rate, 2.5 m*M* calcium chloride, 109 m*M* sodium chloride, 4.5% w sucrose, 0.3% tri(n-butyl)phosphate and 1% Tween-80. The mixtu was incubated with stirring at 25 °C for 17 h prior to purification factor VIII by ion exchange chromatography [27]. 14 ml DEA Toyopearl 650M (TosoHaas GmbH, Stuttgart, Germany) was pack into a 10-mm diameter column (C10/20, Pharmacia) using 2 *M* soc um chloride and equilibrated with 110 ml of buffer containing 1 m*M* glycine, 16 m*M* lysine, 10 m*M* trisodium citrate, 1 m*M* calciu chloride and 110 m*M* sodium chloride at pH 7.0. The SD-treated fa tor VIII solution was applied to the column followed by 30 ml equi bration buffer and 45 ml equilibration buffer raised to 145 m*M* soc um chloride, all at a flow rate of 1.3 ml/min. Factor VIII was th eluted using 26 ml of equilibration buffer raised to 250 m*M* sodiu chloride, at a flow rate of 0.8 ml/min. A sample was also taken of t flowthrough (unadsorbed fraction) from the starting material, as th fraction is used in the manufacture of fibrinogen (fig. 1).

Membrane Filtration of Factor VIII (Step 16)

Microsomal inoculum (9.7 ml) was added to factor VIII solutic (105.4 ml) with a total protein content of 0.43 g/l, which had been fo mulated in elution buffer (step 15) plus 0.1% w/v sucrose. Two mer brane filters (47-mm discs, Durapore 0.45 µm and 0.22 µm, Mill pore) were assembled in series and primed with 12 ml formulatic buffer, prior to filtration of factor VIII solution at a rate of 6.4 ml/mi Filtration was halted, due to blockage of the filters, after 40 ml of t 'spiked' factor VIII solution had been processed. The analysis of th step was therefore based on the 40 ml of starting material that was a tually filtered.

All samples taken were adjusted to approximately pH 7.0, if ne essary, and store at ≤ –70 °C pending analysis. Negative control san ples consisted of samples of each starting material taken prior to t addition of microsomal inoculum and samples of fractions obtaine from equivalent processing carried out without addition of micros mal inoculum.

Western Blot Determination of PrP^{Sc}

In order to reduce the background signals in the Western blots, a process samples were ultracentrifuged to pellet the PrP^{Sc} fibrils, fo lowed by re-suspension in PBS. For Western blot analysis, two 50-µ aliquots of each sample were incubated at 37 °C for 1 h, one in t presence of protease-K (1 µl ; 50 µg/ml) and the other serving as a undigested control. After the addition of an equal volume of SD boiling mix (10% SDS; 50 m*M* Tris/HCl, pH 6.5; containing β-me captoethanol), antigens were denatured by incubation in boiling wat for 3 min. 30 µl of each sample was then loaded onto a Tris-base 12% polyacrylamide gel (Bio-Rad), and the gels electrophoresed 150 V until the blue marker was ~1 cm from the bottom of the ge Pre-stained molecular weight standards were run on the gel to facil tate accurate identification of the size of the immunolabelled band Proteins were transferred to Imobilon-P using a semi-dry blotting pr cedure, and the membranes washed in TTBS (25 m*M* Tris-HCl, pl 7.6, 0.05% Tween 20 and 0.5 m*M* NaCl) for approximately 10 mi followed by blocking with TTBS containing 5% skimmed milk pow der (Marvel) for approximately 1 h. PrP protein was detected usin the monoclonal antibody 3F4 specific for hamster PrP [28]. This anti body (supplied by Senetek PLC) reacts with residues 109–112 Pr