by the intracerebral route (i.c.). Intravenous (i.v.) administration is believed to result in a 10-fold reduction in infectivity compared to the i.c. route, whilst a species barrier may result in up to a 10^3-fold reduction in infectivity (Bader *et al.*, 1998).

To determine the partitioning behaviour of a TSE agent across a preparative process or an individual process step, measurements of the concentrate of infective agent ($ID_{50}\,mL^{-1}$) and the respective process volumes can be used to calculate a TSE agent reduction factor (RF) where

$$RF = \text{total } ID_{50} \text{ before processing} \div \text{total } ID_{50} \text{ after processing.}$$

The same units of measurement are used in the numerator and the denominator and therefore the RF is a dimensionless number which, as values can be high, is often expressed in the logarithmic ($\log_{10}$) form.

Protein precipitation technology

The very low aqueous solubility of PrP^{Sc} suggests that abnormal prion proteins will generally tend to partition into the solids phase in a precipitation process and be separable from proteins which remain in solution and to copurify with proteins which partition into the solids phase.

Cryoprecipitation. The solids phase which forms when plasma is thawed is known as cryoprecipitate; it is where the least soluble proteins tend to precipitate (i.e. fibrinogen, fibronectin, factor VIII, von Willebrand factor) and is the first stage in the overall fractionation process (Fig. 1).

Some information concerning the partitioning behaviour of TSE agents during cryoprecipitation is available from the work of Brown *et al.* (1998) who reported that infectivity from a mouse adapted strain of a human TSE, Gerstmann–Sträussler–Scheinker syndrome (GSS), was found to concentrate in the precipitate phase with an infectivity about one order of magnitude greater in cryoprecipitate than in the plasma from which it was prepared. A similar observation has been reported by Petteway *et al.* (1998), using an immunochemical method of analysis, who found that 90% of hampster adapted PrP^{Sc} (strain 263K) added to plasma partitioned into cryoprecipitate.

Ethanol precipitation. The iso-electric precipitation of proteins in the presence of ethanol forms the basis of cold-ethanol (Cohn) fractionation which is used in the preparation of albumin and immunoglobulins. A number of successive precipitation steps are employed, in which the least soluble proteins are precipitated first and the more soluble proteins being concentrated into later fractions (Cohn *et al.*, 1946). Brown *et al.* (1998) have reported that GSS infectivity partitioned preferentially into the earlier fractions in a cold-ethanol (Cohn) fractionation scheme. Similar observations were reported by Petteway *et al.* (1998) who observed a 10-fold reduction of PrP^{Sc} (263K) in the supernatant following a Cohn fraction I precipitation, a $\geq 3\times10^2$-fold reduction over a Cohn fraction III precipitation and $\geq 3\times10^2$-fold reduction across a Cohn fraction IV precipitation.

Iso-electric precipitation in the presence of ethanol was used as an early purification step in the manufacture of human growth hormone where, in a scrapie clearance study, mouse adapted PrP^{Sc} (strain ME7) in the supernatant was reduced from $3\times10^7\,ID_{50}\,mL^{-1}$ to $\leq 3\,ID_{50}\,mL^{-1}$ after a clarifying precipitation at pH 6 followed by ethanol precipitation (10% ethanol, pH 4·8) of the growth hormone (Taylor *et al.*, 1985). As the growth hormone was precipitated, any CJD infectivity present would have been expected to copurify with the product at this stage of manufacture.

Other solubility methods. Petteway *et al.* (1998), using an immunochemical method of analysis, have reported a 3×10^2-fold reduction of PrP^{Sc} (263K) in a precipitation step used in the preparation of a factor VIII concentrate from cryoprecipitate.

PEG precipitation has also been the subject of a scrapie clearance study, with an overall $\geq 4\times10^5$-fold reduction of PrP^{Sc} (ME7) infectivity being measured following filtration of the supernatant which remained after a light surface phase and two PEG precipitates had been discarded (M. Macnaughton and A. Shepherd, personal communication, April 1997).

In the preparation of aprotinin from bovine lung, Golker *et al.* (1996) studied the distribution of PrP^{Sc} (ME7) infectivity during a water/salt/methanol extraction of lung tissue. The concentration of PrP^{Sc} in the extract was reduced from $10^7\,ID_{50}\,mL^{-1}$ to $\leq 50\,ID_{50}\,mL^{-1}$ (mean of two runs), giving a mean reduction factor across the methanol extraction process of $\geq 2{\cdot}5\times10^4$.

Adsorption/desorption technology

As abnormal prion proteins are strongly membrane bound (Stahl *et al.*, 1990), possess hydrophobic and hydrophilic domains (Bolton *et al.*, 1987) and tend to adhere to surfaces, it is probable that they will interact with chromatographic (Foster, 1994; Burnouf, 1995) and filtration (Meltzer, 1987) media used in plasma fractionation. In these circumstances, a high degree of separation from abnormal prion protein may be possible where a plasma protein does not adsorb to a given matrix. Where a plasma protein is adsorbed, separation from abnormal prion protein will be dependent on the relative strength of binding of each of the macromolecular components.

The separation of PrP^{Sc} infectivity from a variety of

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Table 1. Reduction of scrapie infectivity (ID_{50}) by chromatographic separations

Method	Product	Scrapie strain	Scrapie reduction factor: unadsorbed fraction	Scrapie reduction factor: desorbed fraction	References
Ion-exchange chromatography					
DEAE-cellulose (anion)	n/a*		$2{\cdot}5\times10^{1}$†	1×10^{2}†	Hunter & Millson, 1964
Q-sepharose (anion)‡	plasma protein	ME7	n/d*	$>2{\cdot}5\times10^{2}$	
SP-sepharose (cation)‡	plasma protein	ME7	n/d	$1{\cdot}6\times10^{2}$	
Resin I (undisclosed)	aprotinin	ME7	n/d	$1{\cdot}6\times10^{5}$	Kozak *et al.*, 1996; Golker *et al.*, 1996
Resin II (undisclosed)	aprotinin	ME7	n/d	1×10^{4}	Kozak *et al.*, 1996; Golker *et al.*, 1996
Ion exchange (undisclosed)	aprotinin	263K	n/d	$1{\cdot}2\times10^{5}$	Blum *et al.*, 1998
Ion exchange (undisclosed)	bovine albumin	263K	n/d	$1{\cdot}6\times10^{5}$	Blum *et al.*, 1998
Hydrophobic chromatography					
Phenyl sepharose‡	plasma protein	ME7	n/d	$>1{\cdot}6\times10^{3}$	
Ion exchange + hydrophobic chrom.					
DEAE-spherodex/LS® + DEA-spherosil/LS®	human albumin	C506/M3	n/d	$3{\cdot}1\times10^{5}$	Grandgeorge *et al.*, 1997
Nonspecific adsorption					
Calcium phosphate	n/a		$>1{\cdot}5\times10^{4}$†	$1{\cdot}4\times10^{2}$†	Hunter & Millson, 1964

* = n/a, not applicable; n/d, not done. ‡ M. McNaughton & A. Shepherd, personal communication, April 1997. † = approximation.

proteins has been studied using anion exchange chromatography, cation exchange chromatography, hydrophobic interaction chromatography, nonspecific adsorption and a number of ion exchange procedures for which the details were not disclosed. The results, summarized in Table 1, demonstrate removal of PrP^{Sc} infectivity by all of these procedures ranging from 10^2-fold to 10^5-fold reduction.

In their study of the Lowry process used to prepare human growth hormone, Taylor *et al.* (1985) observed a 10-fold reduction in PrP^{Sc} (ME7) infectivity after filtration through a 0·45-μm cellulose acetate membrane, even though the membranes were pretreated to prevent adsorption. Taylor *et al.* (1985) also noted that 'substantial amounts of scrapie infectivity can be lost by adsorption to membrane filters', and therefore a similar degree of removal of abnormal prion protein might also be expected to occur in comparable membrane filtration operations used in plasma fractionation.

Extrapolation of existing knowledge to plasma fractionation processes

From data available on the behaviour of PrP^{Sc} in a variety of bioprocess operations, it is possible to estimate how a TSE agent might be expected to partition across similar unit operations used in the preparation of pharmaceutical protein products from human blood plasma. Where removal of a TSE agent by a particular plasma fractionation procedure is anticipated, a value for the reduction factor has been assigned (Table 2) using conservative values from a relevant study. For process operations not listed in Table 2, it is assumed that abnormal prion protein will copurify with the plasma product being prepared.

Precipitation. From the information available the causative agents of TSEs would be expected to partition into the solids phase during protein precipitation operations. Where the solubility of a TSE agent is zero and the product protein remains in solution, separation of the product from the TSE agent will be possible. The degree of separation achieved will be influenced by the effectiveness of the technology used to separate the solid phase from the liquid, with a greater assurance of TSE agent removal where two solid–liquid separation operations are carried out in series (e.g. centrifugation followed by filtration).

Adsorption/desorption. Studies concerning a number of biopharmaceutical products have demonstrated that

Table 2. Estimated ability of bioprocess technologies to remove TSE agents

Process technology	Recovered fraction	Estimated TSE agent reduction factor
Precipitation		
Cryoprecipitation	Supernatant	10^1
Cohn fraction I	Supernatant	10^1
Other Cohn fractions	Supernatant	10^2
Other precipitation methods	Supernatant	10^1–10^2
Adsorption chromatography		
Packed bed	Desorbed	10^2
Packed bed	Nonadsorbed	10^1
Suspension	Nonadsorbed	10^1
Adsorptive filtration		
Depth filter (mixed bed)	Non-adsorbed	10^2
Depth filter (single bed)	Non-adsorbed	10^1
Membrane filter (cellulose acetate)	Non-adsorbed	10^1

PrP^{Sc} infectivity binds to a range of adsorbents, resulting in its partial or complete removal from the manufacturing process (Table 1). These data suggest that similar procedures in plasma fractionation processes should also be capable of removing a TSE agent from the product stream to a comparable extent.

In these circumstances the TSE agent reduction factor will be determined not only by the relative binding characteristics of the macromolecules, but also by the unit capacity of the adsorbent and by the technology employed for contacting the process solution with the adsorptive media, with flow through a packed bed (column) being expected to afford the highest degree of separation.

Separation of PrP^{Sc} occurred with all of the adsorbents examined (Table 1), despite the use of different ligands, matrices and principles of adsorption. Therefore, the outcome was not determined by a single well-defined property of PrP^{Sc} (e.g. charge), but must have involved either a number of different properties which caused PrP^{Sc} to be adsorbed in all of these different circumstances, or some form of binding which was common to all of these different methods.

If it is assumed that the reduction in PrP^{Sc} (ME7) infectivity by membrane filtration observed by Taylor *et al.* (1985) was a result of adsorption of the TSE agent to the membrane, rather than removal by a sieving mechanism, then TSE agent removal would be expected to be influenced by the chemical nature of the membrane. Therefore, a TSE agent reduction factor (Table 2) has been assigned only to SNBTS membrane filtration steps (Fig. 1) where the chemical composition of the filter is comparable to that used by Taylor *et al.* (i.e. cellulose acetate).

DISCUSSION

The development of methods for the elimination of viruses such as the human immunodeficiency virus (HIV) and the viruses of hepatitis B (HBV) and hepatitis C (HCV) from labile plasma products (Cuthbertson *et al.*, 1991; Foster *et al.*, 1997) has been a significant achievement. Procedures that can inactivate viruses are especially important; however, the removal of viruses by separations technology has also contributed to product safety. Viral contaminants can be preferentially removed by precipitation (Budnick *et al.*, 1994), depth filtration (Bhattacharya *et al.*, 1996) and chromatography (Burnouf, 1993; Darling & Spaltro, 1996), with removal by cold ethanol (Cohn) fractionation contributing significantly to the freedom from hepatitis transmission of human albumin (Hoofnagle & Barker, 1976) and immunoglobulin (Pennell, 1957). These technologies would also be expected to preferentially remove causative agents of TSEs (Blum *et al.*, 1998). Whether or not complete removal of a TSE agent is achievable is less certain, especially with less purified or less processed products. It is important in this context to appreciate that a very high reduction factor would not necessarily indicate that an infectious agent had been removed completely by the process concerned. Although the reduction factor provides a useful indication of the capacity of process operations to remove an infectious agent, additional information is required to determine whether or not some residual (resistant) infectious material may remain in a process stream after a step in question.

In precipitation processes it is necessary to define the solubility of the infectious agent under the precipitation

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conditions being employed. Unless the solubility is zero, then a quantity of the agent will remain in solution. Brown *et al.* (1998) were able to detect PrP^{Sc} infectivity in a fraction V precipitate prepared from normal human blood which had been 'spiked' with hamster adapted scrapie (263K), but with a 10^6-fold reduction from the original titre in the whole blood. Whether this small degree of infectivity resulted from a small proportion of PrP^{Sc} remaining soluble prior to the fraction V precipitation or if there was incomplete removal of earlier solids fractions is unclear. Taylor *et al.* (1985) were unable to detect PrP^{Sc} (ME7) in the supernatant following precipitation of human growth hormone with 10% ethanol at pH 4·8. However, the limit of detection quoted was 0·5 $\log_{10} ID_{50}\,mL^{-1}$ (i.e. $3\,ID_{50}\,mL^{-1}$) so it is possible that this concentration of PrP^{Sc} (ME7) could have been soluble and remained undetected in solution.

Different considerations apply to methods involving adsorption (and desorption) as the reduction factor should largely be indicative of whether or not a separation can be achieved and what the capacity of a process operation would be. The potential for interference by the TSE agent inoculum being added to challenge a process step must also be considered as constituents of a brain homogenate used as a source of PrP^{Sc} might either occupy adsorption sites which would otherwise be available for the binding of the TSE agent or, alternatively, might provide specific binding sites for PrP^{Sc} that would not otherwise exist. Where adsorption technology is employed for TSE agent removal then to avoid cross-contamination of subsequent batches it will be necessary either to use new adsorption media on each occasion or to sanitize media and equipment effectively before re-use.

Most TSE agent clearance studies have involved the addition of a brain homogenate to the process solution to be studied. How accurately this model represents the behaviour of endogenous TSE agents in human plasma is an important question. Brown *et al.* (1998) have reported two partitioning studies, one using human blood spiked with scrapie (263K) infected hampster brain and the other using murine blood obtained from mice infected with a strain of a human TSE (GSS). Comparable results were obtained in the fractionation of plasma from each experiment, indicating that the use of brain homogenate reasonably represented the behaviour of an endogenous TSE agent. Whether or not this finding will apply equally to processes or experimental procedures other than those employed by Brown *et al.* (1998) remains to be determined.

To appreciate the significance of the magnitude value of a reduction factor over an individual stage, it is necessary to relate its value to the potential quantity of the infectious agent that requires to be removed or inactivated. For example, where there is a high concentration of a virus in a plasma donation (e.g. HIV, HBV, HCV, B19 parvovirus) then a relatively high degree of reduction (e.g. 10^4-fold) may be required over individual process steps to assure product safety (Darling & Spaltro, 1996). However, where the concentration of the infective agent is relatively low (e.g. TSE agents in plasma) then a small degree of reduction may be significant (Brown, 1998).

Whether or not the individual reduction factors for each step in a process (Fig. 1) can be added together to provide a notional overall reduction factor across a complete process (Table 3) is dependent on the properties and state (e.g. degree of aggregration) of the infectious agent, the principles of the separation technologies concerned, the conditions at each step, the relative positions of different technologies within a process and other factors which might limit the effectiveness or capacity of a particular step or technology (Hageman, 1991). For TSE

Table 3. Estimated TSE agent reduction for each SNBTS plasma product

Product	No. process steps contributing to TSE agent reduction			Sum of estimated TSE agent reduction factors
	Precipitation	Adsorption (gel)	Adsorption (filter)	
Albumin (Alba®)	3	1*	5	10^{13}
Immunoglobulins	2	1*	5	10^9
Factor IX (HIPFIX®)	1	3	–	10^7
Thrombin	1	2	2	10^7
Fibrinogen	1	2	2	10^5
Factor VIII (Liberate®)	1	2	–	10^4
Factor II, IX, X (DEFIX®)	1	1	–	10^3

* Step applied only to 1/3rd of plasma pools and discounted in summation of reduction factors.

agents, where different operating conditions are employed in a series of successive steps, then each removal step is generally, but not always, regarded as additive (Rohwer, 1996). Where the same or similar step is used more than once, reduction factors may be additive if TSE agent removal is limited by the capacity of the step, but not where an equilibrium relationship (e.g. solubility of the TSE agent) is limiting.

Much remains to be learned concerning the physico-chemical properties of TSE agents in general (Edenhofer *et al.*, 1997) and nvCJD in particular. In the absence of such data it is inevitable that uncertainty will exist over the ability of particular process steps, either individually or in combination, to fully remove any nvCJD agent which may be present. In these circumstances the availability of a number of process steps which would be expected to remove a TSE agent by different mechanisms will provide a greater assurance of product safety than reliance on either a single step or a single mechanism of removal. The fact that plasma products are manufactured via a number of process steps which would be expected to operate in a complementary manner may be of particular importance in this regard.

POSSIBLE nvCJD CONTENT OF PLASMA PRODUCTS

In order to estimate the possible nvCJD content of a plasma product it is necessary to first estimate the nvCJD content of the starting plasma pool, secondly to calculate the quantity of nvCJD infectivity remaining after processing and thirdly to consider how this material may be distributed in the vials or bottles of the dispensed product.

To determine the quantity of nvCJD infectivity that could potentially be present in a plasma pool, it is necessary to know the dose of nvCJD needed to transmit infection from human to human by intravenous or intramuscular administration, the number of infectious doses present in the plasma of an infected blood donor and the number of infected donations present in the plasma pool.

There are as yet no data available on the nvCJD content ($ID_{50}\,mL^{-1}$) of human blood or plasma. However, as nvCJD is believed to be human BSE (Almond & Pattison, 1997), then bovine data probably represent the best information currently available for the purpose of estimating the infectivity of blood from a person infected with nvCJD. BSE was not detected in the blood or serum of infected cattle, by i.c. injection into mice (Kimberlin, 1996). However, the limit of detection in these studies was 25 $ID_{50}\,mL^{-1}$ and, given the species barrier involved, the within-species infectivity could have been as high as 25 000 i.c. $ID_{50}\,mL^{-1}$. Correction for the route of infusion (from i.c. to i.v.) could give a within-species infectivity of blood of up to 2500 i.v. $ID_{50}\,mL^{-1}$.

TSE infectivity has been found to be associated with white blood cells (Kuroda *et al.*, 1983) and consequently the separation of plasma from the cellular components would be expected to result in a significant proportion of a TSE agent being removed. If a 10-fold reduction is assumed in the routine centrifugal separation of plasma from whole blood (Brown *et al.*, 1998), then the estimated concentration of nvCJD in an infective plasma donation would be 250 i.v. $ID_{50}\,mL^{-1}$. With these assumptions, a single infective 300-mL donation of plasma could contain a total nvCJD infectivity of up to $7{\cdot}5\times10^4$ i.v. ID_{50}. Further reduction of the white cell content of plasma by leucofiltration (Rider *et al.*, 1998) may also reduce the nvCJD content, but as the degree of reduction is uncertain, any contribution that may be afforded by this technology has been discounted.

To examine the possible implications of an epidemic of nvCJD in the UK it is necessary to calculate the degree of product contamination that could result, in theory, from processing a contaminated pool of plasma. It has been estimated that up to 80 000 people in the UK could have been infected by BSE (Cousens *et al.*, 1997). This represents a cumulative incidence of 1500 per 10^6 of the UK population and, although there is as yet no evidence to support such a figure, this particular prediction may be taken as a 'worst case' scenario for the purposes of theoretical calculations. If plasma from UK donors is contaminated to this extent then the concentration of nvCJD in a plasma pool could be $3{\cdot}7\times10^{-1}$ i.v. $ID_{50}\,mL^{-1}$. Therefore, for a plasma product to be infective in this scenario, the nvCJD agent in the plasma pool must be concentrated into one or more of the products in question.

From the information available on the behaviour of TSE agents, the opposite might be expected during plasma fractionation, in that all plasma products are manufactured using procedures which would be expected to remove TSE agents to some extent. To illustrate this, the impact of a 0·15% incidence of infection (i.e. a worst case scenario) has been calculated in terms of the total infectivity that would be present in the plasma pool, the purified product pool prior to dispensing and in each vial of dispensed product for current products manufactured by the SNBTS and for some earlier products (Table 4), which are included as it is conceivable that nvCJD may have been present subclinically in the blood donor population since the early to mid-1980s (Cousens *et al.*, 1997).

If it is assumed that the final product pool is homogenous and that nvCJD infectivity is distributed equally amongst all of the vials of product dispensed, it can be calculated that no vial of any current product would contain an infectious dose (ID_{50}). It should be noted that these theoretical values were determined assuming a high incidence of nvCJD in the human population (1500 per

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Table 4. Theoretical estimates of the quantity of nvCJD in products prepared from pooled plasma where 0·15% of donations contain nvCJD

		Total nvCJD (i/v. ID_{50})		
SNBTS product	Volume plasma per batch (L)	In plasma pool*	In product pool pre-dispensing	In final vial†
Albumin, 4·5% (Alba®)	2000	$7{\cdot}5\times10^{5}$	$7{\cdot}5\times10^{-8}$	$3{\cdot}0\times10^{-11}$
Albumin, 20% (Alba®)	2500	$9{\cdot}7\times10^{5}$	$9{\cdot}7\times10^{-8}$	$3{\cdot}4\times10^{-11}$
IgG i/m	1500	$6{\cdot}0\times10^{5}$	$6{\cdot}0\times10^{-4}$	$3{\cdot}8\times10^{-9}$
IgG i/v	2000	$7{\cdot}5\times10^{5}$	$7{\cdot}5\times10^{-4}$	$3{\cdot}7\times10^{-7}$
Thrombin	3000	$1{\cdot}1\times10^{6}$	$2{\cdot}2\times10^{-3}$‡	$1{\cdot}9\times10^{-6}$
Factor IX HIPFIX®	2700	$1{\cdot}0\times10^{6}$	$1{\cdot}0\times10^{-1}$	$1{\cdot}2\times10^{-4}$
Fibrinogen	2000	$7{\cdot}5\times10^{5}$	$7{\cdot}5\times10^{0}$	$6{\cdot}2\times10^{-3}$
Factor VIII Liberate®	4000	$1{\cdot}5\times10^{6}$	$1{\cdot}5\times10^{2}$	$9{\cdot}2\times10^{-2}$
FII, IX, X DEFIX®	3000	$1{\cdot}1\times10^{6}$	$1{\cdot}1\times10^{3}$	$6{\cdot}2\times10^{-1}$
Factor VIII (Z8)§	1000	$3{\cdot}7\times10^{5}$	$3{\cdot}7\times10^{3}$	$3{\cdot}7\times10^{0}$
Factor VIII (NY)§	1000	$3{\cdot}7\times10^{5}$	$3{\cdot}7\times10^{4}$	$2{\cdot}7\times10^{1}$

* Based on nvCJD infectivity of 250 i.v. $ID_{50}\,mL^{-1}$ in plasma from each infected donation (300 mL). † Assumes an even distribution of nvCJD amongst all vials in a batch of product. ‡ Only about 2% of plasma pool processed to thrombin. § Products discontinued in 1992 (Z8) and 1986 (NY).

10^{6}), a relatively high infectivity of nvCJD in plasma (i.e. 250 i.v. $ID_{50}\,mL^{-1}$) and generally low values for the TSE agent process reduction factors (Table 2).

However, these calculations also involved a number of assumptions concerning process reduction factors that were extrapolated from a small number of studies that were themselves based on animal model systems not necessarily predictive for the human situation. Therefore, it is inevitable that uncertainty remains over whether or not there may be a risk of nvCJD being transmitted by any of the plasma products assessed. To obtain a more certain estimate of risk it will be necessary to determine the infectivity of the causative agent of nvCJD, its prevalence in the UK blood donor population and the effectiveness of plasma fractionation processes in removing TSE agents using appropriate measurements.

CONCLUSIONS

All of the available evidence concerning the properties and behaviour of the causative agents of TSEs suggests that a number of the bioseparations technologies used in the manufacture of human plasma products should have a potential to remove the causative agent of nvCJD. For each SNBTS product, the estimated potential for nvCJD removal involves processing by multiple unit operations and different principles of separation, both of which provide a greater degree of assurance than would be obtained with reliance on either a single step or a single mechanism of separation.

This assessment suggests that should there be a major epidemic of nvCJD in the UK, then most SNBTS plasma products prepared from plasma collected in the UK should have a very low risk of being contaminated. Nevertheless, many uncertainties remain and it will be necessary to establish the accuracy of these estimates in appropriate validation studies. Such studies should also indicate whether or not adsorption or precipitation technologies used in plasma fractionation could be exploited further to provide an increased capacity for the removal of human agents of TSE.

ACKNOWLEDGMENTS

I am indebted to Dr Malcolm McNaughton and Ms Ailsa Shepherd of Inveresk Research and to their client for providing data from an unpublished validation study of scrapie removal. I am also grateful to Kirsty Glass

and Druscilla Rodger for their skilled assistance in the preparation of this manuscript.

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