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ORIGINAL PAPER

Prion-removal capacity of chromatographic and ethanol precipitation steps used in the production of albumin and immunoglobulins

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Vox Sanguinis

Background and Objectives Although there is no epidemiological evidence to suggest that classical Creutzfeldt–Jakob disease (CJD) is transmitted through blood or blood products, the variant form (vCJD) has been implicated in transmission via packed red blood cells. The potential threat of the infectious agent contaminating plasma pools has led to manufacturing processes being examined for capacity to remove prions. The objective of these studies was to examine the prion-removal potential of the chromatographic purification and ethanol precipitation steps used to fractionate immunoglobulins and albumin from human plasma.

Materials and Methods Western blot assay was used to examine the partitioning of proteinase K-resistant scrapie prion protein (PrP^{sc}) over DEAE Sepharose, CM Sepharose and Macro-Prep High Q chromatographic columns, utilizing microsomal scrapie 263K spiked into each scaled down feedstream and assayed after each chromatographic step. In further studies, bioassay in C57 black mice was used and spikes of 10 000 g clarified brain homogenate of scrapie ME7 were added to feedstreams before sequences of scaled down chromatographic or Cohn fractionation process steps.

Results The microsomal spiking study with Western blot detection demonstrated substantial partitioning of PrP^{sc} away from the target proteins in all ion exchange chromatographic steps examined. The $\log_{10}$ reduction factors (LRF) across DEAE Sepharose and CM Sepharose columns for albumin were ≥ 4.0 and ≥ 3.0 respectively. The reductions across DEAE Sepharose and Macro-Prep High Q for intravenous immunoglobulin were 3.3 and ≥ 4.1 respectively. Bioassay demonstrated LRFs of ≥ 5.6 across the combination of DEAE Sepharose and CM Sepharose columns in the albumin process and ≥ 5.4 across the combination of DEAE Sepharose and Macro-Prep High Q columns in the intravenous immunoglobulin process. Bioassay studies also demonstrated a LRF of ≥ 5.6 for immunoglobulin produced by Cohn fractionation.

Conclusions Using rodent-adapted scrapie as a model, the studies indicated that ion exchange chromatography, as well as Cohn immunoglobulin fractionation have the potential to effectively reduce the load of TSE agents should they be present in plasma pools.

Table of Contents Ion exchange columns used for production of human albumin and immunoglobulins, as well as Cohn immunoglobulin fractionation, effectively reduce the load of TSE agents should they be present in plasma pools.

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Key words: bioassay, chromatography, prion, scrapie, transmissible spongiform encephalopathy, Western blot.

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Introduction

The outbreak of the variant form of Creutzfeldt–Jakob disease (vCJD), linked to a bovine spongiform encephalopathy (BSE) in the UK, and the propensity of this form to accumulate in peripheral lymphoid tissues, has raised the theoretical possibility of blood-borne transfusion of the vCJD agent. Experimental studies in a sheep model in which BSE was transmitted via blood transfusions [1] demonstrate proof of principle for this possibility. It is probable that transmission has occurred in humans with the report of vCJD in a blood transfusion recipient 6·5 years after receiving red blood cells from a presymptomatic vCJD donor [2]. This report led to the identification of 20 U of plasma from individuals who later developed vCJD that were pooled to produce fractionated products used to treat thousands of recipients: to date, no cases of vCJD have been identified in recipients of these fractionated plasma products.

Evidence that vCJD may be transmitted by red blood cell transfusion followed the post-mortem detection of proteinase K-resistant scrapie prion protein (PrP^{sc}) in the spleen and lymph node of a patient who died of other causes, having previously receiving a red blood cell transfusion from a donor that subsequently developed vCJD [3]. More recently, the UK National CJD Surveillance Unit has announced a 'probable' third case of transfusion-related vCJD, in which the patient (who is still living) developed symptoms of vCJD about 8 years after receiving a blood transfusion from a donor who developed symptoms of vCJD about 20 months after donating this blood [4]. In contrast to vCJD, classical CJD transmission by blood transfusion has never been reported in humans [5].

The potential risk of vCJD transmission led producers of plasma products to examine the prion-removal capacity of their fractionation processes [6–10]. A difficulty with accurately modelling the removal of blood-borne infectious prions from plasma processes is identifying the form of 'spiking' material that best represents what might be present in blood. The best representation of blood-borne infectivity is the use of blood ex-sanguinated from test animals with clinical TSE [11,12]; however, the low infectivity level found in blood does not enable a high infectivity challenge of plasma fractionation processes. TSE-infected brain material offers much higher levels of infectivity and a variety of preparations have been reported. Ideally, a range of different spiking materials would be tested on each process step [9]; however, in practice, investigators have selected one or two preparations for their experiments because of practical limitations including the many test animals required for bioassays.

Rodent-adapted scrapie has been used extensively as a model for the study of prion partitioning during plasma processing steps [6,10,13,14]. The incubation period of murine-adapted scrapie strain ME7 has been characterized as 171 ± 2 days in C57 black mice [15] which carry the Sinc s7 gene for short scrapie incubation time. The hamster-adapted 263K strain has also been widely used.

Most published studies investigating the prion-removal potential of processes used to purify albumin and immunoglobulins (IgG) from plasma have focused on studies of Cohn fractionation, and comparatively little information has been published on prion-removal potential of chromatography-based processes. The prion-removal potential of the chromatographic portion of processes used to fractionate intravenous immunoglobulins and albumin [16] was examined using two methodologies. The first study utilized a microsomal preparation of hamster-adapted scrapie 263K, detected by Western blot. This study focused on clearance over individual columns in the processes.

The second study used mouse-adapted scrapie ME7, with detection by bioassay in C57 black mice. This study used a 10 000 g clarified homogenate spiking material, which is characterized as containing both microsomal and 'soluble PrP^{sc}' [17], to assess overall removal capacity of the ion exchange chromatography steps in the production process. The TSE infectivity reduction potential of the Cohn–Oncley [18,19] process employed in manufacturing hyperimmune immunoglobulins was also examined.

Materials and methods

Experimental design

All parameters of the industrial processes were scaled down to give accurate representations of chromatography and other process conditions. New chromatography gels were used for all experiments. Columns sizes were held at the same height as production columns, however, with smaller diameter. The scale-down factor for the DEAE and CM Sepharose columns was 1 : 5625, and for the Macro-Prep High Q column was 1 : 14 667. The number of column volumes of buffers was exactly the same as the production processes; protein loadings and linear flow rates were set at the maximum allowable in the production processes. It was required that all buffers and column conditions achieved the same pH, conductivity and height equivalent of a theoretical plate (HETP) limits as are applied to the production process. Control runs were performed with feedstreams spiked with uninfected brain preparations as described below. Extensive testing of all buffers and protein eluates determined whether the control runs accurately represented the industrial processes, as previously described [20]. Due to the limited scope of assays that could be performed in containment conditions for the TSE spiked runs, the validity of the TSE spiked run was assured by following exactly the scale-down conditions used in the control runs.

Experiments using microsomal 263K spiking and Western Blot assay

Preparation of microsomal inoculum

Brain homogenate from hamsters without disease, or in the late clinical stage of infection with hamster adapted scrapie (strain 263K), was used to prepare a microsomal fraction as described [21]. Briefly, crude brain homogenate (10% wt/v) was prepared by Dounce homogenization of brains in phosphate-buffered saline (PBS). This was pelleted at 10 000 *g* for 7 min to remove nuclei, unbroken cells and mitochondria. The microsomes remaining in the supernatant were then pelleted by centrifugation at 100 000 *g* for 90 min, followed by resuspension in PBS.

DEAE Sepharose chromatography

De-lipidated and euglobulin (non-IgG globulins)-depleted Supernatant 1 (SN1) was obtained from the production plant, and 135 ml was 'spiked' at 10% v/v with microsomal control or scrapie 263K and sampled (Fig. 1). DEAE Sepharose™ Fast Flow (DEAE Sepharose) was obtained from GE Healthsciences, Uppsala, Sweden. A 17·5 cm bed height column was equilibrated with 10 mM sodium acetate (NaAc) at pH 5·2, and one-third of the spiked material was loaded. Following loading, the column was washed with 10 mM NaAc buffer and protein elution was monitored by ultraviolet (UV) absorption at 280 nm. The non-retained crude immunoglobulin was collected until the onset of the second peak, in which transferrin was eluted.

The 10 mM NaAc wash was continued until the elution of the transferrin peak was complete. Albumin was then eluted with approximately 2·5 column volumes (CV) of 25 mM NaAc buffer. The column was regenerated with 2 CV of 150 mM NaAc, pH 4·0. The loading and elution cycle was repeated a further two times to load the entire starting volume as per the production process, before regeneration and sanitization in reverse flow with 1 CV of 0·5 M NaCl, 1 CV of 1 M NaOH and 2·5 CV of 150 mM NaAc. All corresponding peak fractions from each cycle (other than the 1 M NaOH eluate) were pooled and assayed by Western blot.

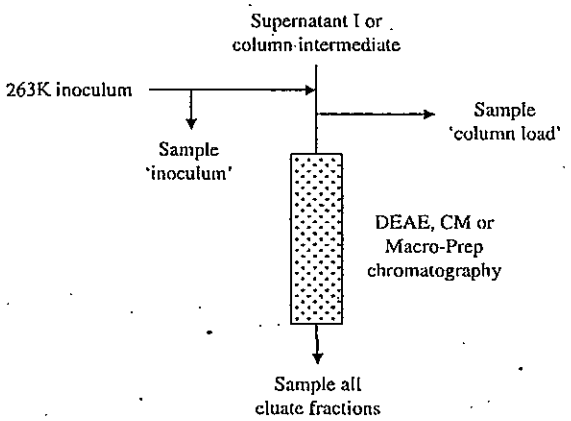

Fig. 1 Flow diagram showing spiking points and sampling points for each column in 263K PrP^{sc} studies. The diagram applies to each of the chromatography columns, as they were each spiked separately.

CM Sepharose chromatography

CM Sepharose™ Fast Flow (CM Sepharose) was obtained from GE Healthsciences, Uppsala, Sweden. A 17·5 cm bed height column was equilibrated with 25 mM NaAc (pH 4·5). Pooled crude albumin from the DEAE Sepharose column was obtained from the production plant, and 150 ml was spiked at 10% v/v with microsomal control or scrapie 263K. After sampling, one-third of the volume was loaded onto the column, and then flushed with 1·8 CV of 25 mM NaAc to elute the unbound proteins. Albumin was then eluted with approximately 3 CV of 110 mM NaAc buffer. The column was regenerated with 1·5 CV of 400 mM NaAc pH 8·0. The loading and elution cycle was repeated a further two times to load the entire starting volume as per the production process, before the column was regenerated and sanitized in reverse flow with 1 CV of 0·5 M NaCl, 1 CV of 1 M NaOH and 2·5 CV of 150 mM NaAc. All corresponding peak fractions from each cycle (other than the 1 M NaOH eluate) were pooled and assayed by Western blot.

Macro-Prep chromatography

Macro-Prep High Q (Macro-Prep) gel was obtained from Bio-Rad, Hercules, CA. A sample of non-retained crude IgG solution from DEAE Sepharose was obtained from an actual production process and 100 ml was spiked at 10% v/v with microsomal control or scrapie 263K. The pH adjusted crude IgG solution was loaded onto a 17·5 cm bed height column that had been equilibrated with 6 CV of 10 mM NaAc, pH 6·2. Two CV of 10 mM NaAc pH 6·2 were used to elute the non-retained immunoglobulins from the column. The column was regenerated with 2 CV of 1·0 M NaCl and 2 CV of 1·0 M NaOH. All column eluates (other than the 1 M NaOH eluate) were assayed by Western blot.

Western blot

Samples were ultracentrifuged at 150 000 *g* for 1 h and the pellet was resuspended in a minimal volume of PBS prior to digestion with proteinase K (Roche, Mannheim, Germany) at 250 μg/ml for 1 h at 37 °C. Digestion was terminated by 1 : 1 addition of sample buffer (125 mM Tris-hydrochloric acid, 20% v/v glycerol pH 6·8, containing 4% w/v sodium dodecylsulphate, 5% v/v 2-mercaptoethanol), then boiled for 3 min. Samples were run on 12% polyacrylamide gels (Bio-Rad, Hercules), and transferred onto Immobilon P (Millipore, Billerica, MA). Membranes were blocked with PBS/Tween 20 (0·05%) containing 5% skim milk and were probed with monoclonal antibody (MAb) 3F4 (Signet

Laboratories, Dedham, MA) at 1/10 000 for 1 h. Rabbit antimouse secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich, St Louis, MO) was used at 1/1000 for 1 h. Blots were developed with ECL reagents (GE Healthcare, Uppsala) and were visualized on Hyperfilm M (GE Healthcare, Uppsala).

After Western blot, the dilution was recorded at which PrP^{sc} could no longer be detected. If PrP^{sc} could not be detected in the neat sample, the total PrP^{sc} (log_{10}) reduction was recorded as '≤'. The formula used to calculate the number of units of PrP^{sc} was: reciprocal of the end point dilution of the sample × the total fraction volume in ml × correction factor applied to control for concentration of the sample following ultracentrifugation. Scrapie reduction was calculated by dividing the total scrapie in the spiked starting material by the total recovered scrapie. Variability of the data could not be assessed, as one Western blot was run per sample.

Experiments using bioassay with ME7 spike

Scrapie inoculum

Scrapie ME7 was incubated in C57 black mice, and brains were harvested from mice in the late clinical stage of infection. The brains were homogenized in PBS at 10% wt/v using a Duall tissue grinder (Kontes, Vineland, NJ), and the homogenate was centrifuged at 10 000 *g* for 30 min to remove cellular debris [17].

Chromatography

All chromatographic conditions described for the Western blot study were replicated for the bioassay study; however, columns were run sequentially without intermediate spiking (Fig. 2). De-lipidated and euglobulin-depleted SNI was obtained from a production batch and was 'spiked' with clarified brain homogenate from control mice or ME7-infected mice to give a final spike concentration of 3·3% v/v. For the TSE spiked run, sample 'ME7 spiked SNI' was taken, and 133 ml of the material was separated on DEAE Sepharose. The albumin and immunoglobulin-containing peaks from each cycle were pooled with the corresponding peaks from each of the three cycles and were further processed on CM Sepharose or Macro-Prep.

The pooled crude albumin was loaded onto a CM Sepharose column. The purified albumin peak eluted from each cycle was pooled with the corresponding peak from the other cycles and was concentrated 10-fold with a Pellicon XL 30 kDa polyethersulphone membrane (Millipore, Billerica), and the sample 'ME7 Albumin' was taken for bioassay.

Crude IgG eluate from the DEAE Sepharose column was loaded onto the Macro-Prep column, and the eluted pure IgG concentrated and diafiltered using a 30 kDa regenerated cellulose YM30 ultrafiltration membrane (Millipore, Billerica).

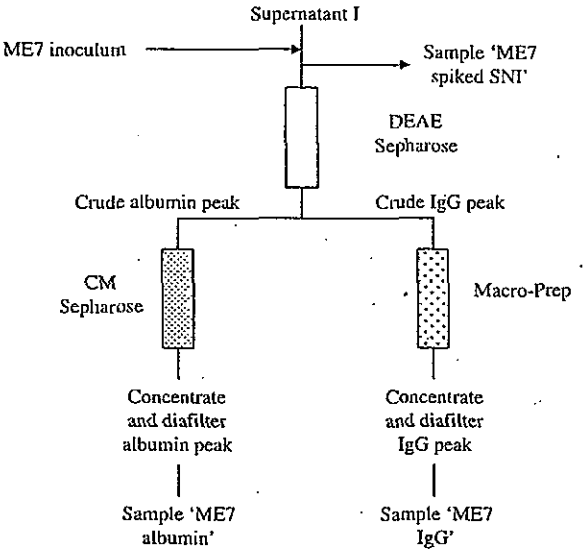

Fig. 2 Flow diagram showing spiking and sampling points for each column in ME7 infectivity studies. One upfront spike was made, with bioassay before and following each purification sequence.

The diafiltered solution was concentrated and the sample 'ME7 IgG' was taken for bioassay.

Immunoglobulin prepared by Cohn fractionation

fraction III supernatant was prepared from cryosupernatant using the Cohn–Oncley process (Fig. 3). Cryosupernatant was cooled to ≤ 1 °C, and was processed either spiked with control homogenized mouse brain at 1 : 29 or with homogenized scrapie mouse brain (ME7, as above) at 1: 29. The sample

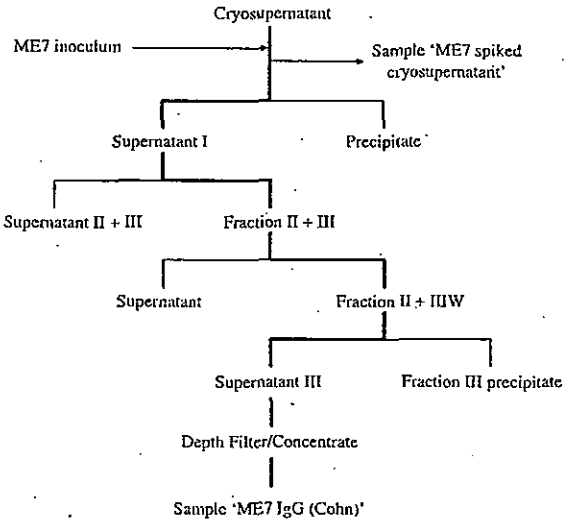

Fig. 3 Flow diagram showing spiking and sampling points for Cohn immunoglobulin ME7 infectivity studies.

'ME7 spiked cryosupernatant' was taken for bioassay, leaving a volume of 316 ml for further processing.

Cold ethanol at ≤ −5 °C was added to achieve a final ethanol concentration of 7·5–8·5% v/v, and Fraction I (fibrinogen) precipitate was separated by centrifugation at 20 000 *g* for 10 min. Cold ethanol was added to the SNI to give a final ethanol concentration of 18·5–22·5% v/v. The mixture was centrifuged at 20 000 *g* for 10 min at −5 ± 1 °C and the Fraction II + III precipitate (immunoglobulin plus lipoprotein) was collected. Sufficient ethanol at ≤ −5 °C was then added to achieve an ethanol concentration of 20·0% v/v, to precipitate immunoglobulins while leaving albumin in solution. Fraction II + IIIW precipitate was separated by centrifugation at 20 000 *g* for 10 min at −5 ± 1 °C. The fraction III precipitate (lipoprotein and IgM) was separated by centrifugation at 20 000 *g* for 10 min at −5 ± 1 °C. Filter aid Diacel 150 (CFF, Gehren, Germany) was added to the fraction III supernatant and filtered through Seitz EK1 disks (Pall, East Hills, NY). The filtrate was adjusted to pH 4·0 and diafiltered at this pH using 10 kDa ultrafiltration membranes. The sample 'ME7 IgG Cohn' was taken for bioassay.

Bioassay

Samples collected from one control run and the TSE partitioning run were used for intracerebral (IC) inoculation of mice. The test materials were subjected to tenfold dilutions in PBS, and weanling C57 black mice (Animal Resources Center, Perth, WA) were IC inoculated with 30 µl of test dilution in sets of five mice per cage. As shown in results in the tables, some dilutions were inoculated into more than one cage, to improve sensitivity when low prion infectivity was expected (given Western blot study results). The study period for the bioassay was 18 months. Mice showing clinical symptoms of scrapie [22] throughout the study or that died within incubation periods consistent with TSE were harvested for TSE evaluation by haematoxylin and eosin staining to detect spongiform change. Further testing using MAb 6H4 (Prionics, Schlieren, Switzerland) for immunohistology and MAb SAF83 (Cayman, Ann Arbor, MI) for Western blot was performed if required. Mice were scored as scrapie positive when clinical signs were confirmed by two or more methods. At the end of 18 months, histology was performed on all surviving mice in dilutions from which scrapie mice had been culled. Histology was also performed on all mice in the lowest dilution for which there were no scrapie cases recorded.

Negative mouse controls within the bioassay component were deemed to be satisfactory when they showed no signs of toxicity over the period of the study or did not contract scrapie over the full study. The 50% end point for infectious dose (ID_{50}) of the bioassay titration was calculated using the Spearman Kärber method [23]. When no infectivity was present in a sample, a 95% probability formula was used to estimate residual infectivity in the sample [24]. The log reduction factor (LRF) of infectious scrapie over the processes was determined by subtracting the scrapie log load of the final concentrated eluates from the log load of the spiked starting material [24].

Results

Scale-down validity

Protein intermediates from control runs showed that the processes were scaled down accurately and were representative of production processes with regard to protein purity, concentration and chemical composition. Chromatographic profiles as shown for the scrapie ME7 spiked scale-down runs accurately represented those obtained from the industrial-scale production process [25]. All buffers and column eluates achieved the same HETP, pH, and conductivity limits as production processes.

Experiments using microsomal scrapie 263K with Western blot detection

Log reduction factors and recovery of PrP^{sc} are shown for the ion exchange columns used for the production of albumin and IVIG (Table 1). All eluate streams from the columns were assayed for PrP^{sc} using Western blot. Substantial partitioning of PrP^{sc} away from the target proteins was achieved in all ion exchange steps examined. The log reductions across the DEAE Sepharose and CM Sepharose for albumin were ≥ 4·0 and ≥ 3·0, respectively. The log reductions across the DEAE Sepharose and Macro-Prep for immunoglobulin were 3·3 and ≥ 4·1, respectively.

Summation of all the PrP^{sc} recovered from all eluates of each column shows that the overall percentage recovery of PrP^{sc} for the DEAE Sepharose, CM Sepharose and Macro-Prep columns are ≤ 0·34, ≤ 1·84 and ≤ 0·03%, respectively. Mass balance was therefore not achieved in all three ion exchange columns up to the final wash with 1 M NaCl. The 1 M NaOH sanitation washes were not studied as NaOH renders PrP^{sc} sensitive to digestion by proteinase K [26], and could lead to aberrant results. The results indicate that some PrP^{sc} was eluted from the DEAE Sepharose and CM Sepharose, but most of the PrP^{sc} was either not recovered or bound to the chromatography gel prior to the NaOH sanitation step.

Scrapie ME7 spike with bioassay detection

Limiting dilution bioassay was used to determine the titre of the spiked supernatant I starting material and the final concentrated eluates from the CM Sepharose and Macro-Prep columns (Table 2). The control mice for all studies remained normal throughout the observation period, indicating that the inocula were non-toxic and that there was no cross-contamination from cages housing TSE-positive mice.

Table 1 Partitioning of PrP^{sc} microsomal fraction during albumin and immunoglobulin purification across ion exchange columns as determined by Western blot

Step/Fraction	Total PrP^{sc} (log_{10})	% PrP^{sc} in fraction	Reduction (log_{10})[b]
DEAE Sepharose™ FF			
Inoculum	3·80		
Column load	4·30	100·00	
Unbound IgG[a]	**0·98**	**0·05**	**3·3**
Transferrin peak	≤ 0·84	≤ 0·03	≥ 3·5
Wash – 10 mM NaAc	≤ 0·92	≤ 0·04	≥ 3·4
Eluted albumin[a]	**≤ 0·32**	**≤ 0·01**	**≥ 4·0**
Wash – 150 mM NaAc	1·63	0·20	2·7
0·5 M NaCl	≤ 0·11	≤ 0·01	≥ 4·2
CM Sepharose™ FF			
Inoculum	3·11		
Column load	3·64	100·00	
Unbound protein	≤ –0·34	≤ 0·03	≥ 4·0
Wash 110 mM NaAc	≤ 0·41	≤ 0·06	≥ 3·2
Eluted albumin[a]	**≤ 0·68**	**≤ 0·13**	**≥ 3·0**
Wash 400 mM NaAc	≤ 0·23	≤ 0·04	≥ 3·4
0·5 M NaCl	1·83	1·58	1·8
Macro-Prep High Q			
Inoculum	3·80		
Column load	4·18	100·00	
Purified IgG (unbound)[a]	**≤ 0·08**	**≤ 0·01**	**≥ 4·1**
Wash 10 mm NaAc	≤ –0·19	≤ 0·01	≥ 4·4
1 M NaCl	≤ –0·07	≤ 0·01	≥ 4·2

[a]Eluates shown in bold are main column eluents used for ongoing processing of albumin or immunoglobulin. All other eluates are waste streams.
[b]If PrP^{sc} (proteinase K-resistant scrapie prion protein) could not be detected in the neat sample, the PrP^{sc} log reduction was recorded as '≥'.

The period between inoculation and days of onset of clinical symptoms for the spiked starting material ranged from an average of 184 days for the neat material through to 252 days in the 10^{-3} dilution. The trend of increasing incubation time with higher dilutions of inoculum was not observed at the higher 10^{-4} and 10^{-5} dilutions, with the single positive mice showing disease onset at 474 and 260 days, respectively. This variability could be due to factors such as IC injection placement or due to the specific nature of the infectivity associated with a single infectious prion unit. TSE was not found in any mice inoculated with the concentrated post-CM Sepharose (albumin) nor in the concentrated post-Macro-Prep eluate (immunoglobulin).

For the study of removal capacity of the Cohn immunoglobulin process, the period between inoculation and days of onset of clinical symptoms for the spiked starting material ranged from an average of 186 days for the neat material through to 347 days in the 10^{-5} dilution (Table 3). The trend of increasing incubation time with higher dilutions of inoculum was not observed at the 10^{-6} dilution, with the single positive mouse showing disease onset at 230 days.

The titre of the ME7 spiked starting material for the chromatography process experiment was found to be 5·4 ID_{50}/ml, with a 95% confidence interval of 4·5–6·3 (Table 4). The LRFs for the combined chromatographic processes were calculated as ≥ 5·6 for the albumin process, and ≥ 5·4 for the immunoglobulin process.

Titration of the ME7 spiked starting material for Cohn fractionation also found the titre to be 5·4 ID_{50}/ml, with a 95% confidence interval of 4·4–6·5. No TSE was found in mice inoculated with the Cohn-purified immunoglobulin, with the LRF for the process calculated as ≥ 5·6.

Table 2 Bioassay of test materials from albumin and immunoglobulin chromatographic processes

Sample	Parameter	Sample dilution 10^0	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}
Control SN1	Mice infected/inoculated incubation period (days)[a]	0/8							
Control Albumin	Mice infected/inoculated incubation period (days)	0/10							
Control IgG	Mice infected/inoculated incubation period (days)	0/9							
ME7 spiked SNI	Mice infected/inoculated incubation period (days)	5/5 184 ± 0	5/5 193 ± 7	5/5 228 ± 14	5/5 252 ± 18	1/5 474	1/5 260	0/5	0/5
ME7 Albumin	Mice infected/inoculated incubation period (days)	0/15	0/5	0/5	0/5				
ME7 IgG	Mice infected/inoculated incubation period (days)	0/20	0/5	0/5	0/5				

[a]Mean ± standard deviation.