Validation of a Prion and Virus Purification Process in the Manufacture of Bovine Thrombin

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ABSTRACT

Background: To further minimize transmission risk of blood-borne viral and prion diseases, manufacturers of therapeutic animal- or human-derived proteins have implemented improved in-process pathogen-removal procedures.

Objectives: To evaluate removal of transmissible spongiform encephalopathy agents (prions) and viruses using Omega 100K VR membrane filtration system (Pall Filtron, Northborough, MA) during manufacture of bovine thrombin.

Methods: Purified bovine thrombin was challenged with a high-titer clarified brain homogenate of the 263K hamster scrapie strain, a model for bovine transmissible spongiform encephalopathy agents, and 4 representative model viruses: bovine parvovirus (BPV), xenotropic murine leukemia virus (XMuLV), pseudorabies virus (PRV), and bovine viral diarrhea virus (BVDV). Using a scaled-down model of the Omega 100K VR filtration procedure, 3 filtration runs were performed on prion-spiked bovine thrombin samples and 2 filtration runs were performed on each virus-spiked bovine thrombin sample. Pre- and post-filtration samples were assayed for prions using a validated Western blot assay and for viruses using validated cell-based infectivity assays.

Post-filtration clearance was assessed by calculating prion and virus log reduction factors (=$\log_{10}$ of ratio of registration prion/virus load and post-filtration scrapie/virus load).

Results: Test prion and viruses were substantially reduced by filtration. Prion log reduction factors were 3.6, >3.7, and >3.7 (runs 1–3, respectively). Average virus log reduction factors were ≥4.92 (PRV), 4.29 (BVDV), 4.26 (XMuLV), and 3.74 (BPV).

Conclusions: The Omega 100K VR filtration step resulted in significant clearance of prions and viruses from purified bovine thrombin. Incorporation of this filtration step into the manufacturing process further improves the biologic safety of bovine thrombin.

INTRODUCTION

Since its approval by the US Food and Drug Administration (FDA) in the late 1970s as a topical aid to hemostasis, bovine thrombin has been used extensively in a variety of surgical procedures, including cardiac, vascular, thoracic, orthopedic, neurologic, general, gynecologic, head and neck, and dental surgery.[1] Used alone or in combination with passive hemostatic agents such as absorbable gelatin sponges,[2] topical bovine thrombin provides rapid and effective hemostasis. Success rates of up to 98% have been reported with its use in combination with absorbable gelatin sponges in cardiac, spinal, and vascular surgeries.[3] Today's bovine thrombin (Thrombin-JMI) is manufactured by King Pharmaceuticals, Inc. (Middleton, WI) and is currently the only non-human source of thrombin available in the United States for therapeutic use.

Progressive purification processes developed by the manufacturer since its introduction to the market have greatly reduced the potential for prion contamination and biocontamination. Like endogenous thrombin, topical thrombin directly participates in the coagulation cascade to produce rapid and effective hemostasis and clotting at the site of bleeding.[3-7] Therefore, topical bovine thrombin is considered to be a common standard for achieving active hemostasis in many surgical procedures.

The inadvertent transmission to humans of pathogens causing blood-borne infectious diseases is a concern with the clinical use of human- or animal-derived therapeutic proteins.[8,9] The risk of transmission of the blood-borne viruses, hepatitis B virus, hepatitis C virus, and human immunodeficiency virus (HIV) is now well recognized with the manufacture of all human- and animal-derived products and a heightened awareness of the need for risk-reduction measures has been realized and implemented by manufacturers. Prions pose the potential risk of transmissible spongiform encephalopathies (TSEs), a group of fatal neurodegenerative diseases linked to misfolded prion proteins.[10] Notable TSEs include Creutzfeldt-Jacob Disease (CJD) in humans, bovine spongiform encephalopathy (BSE) or "mad cow disease" in cattle, and scrapie in sheep.[10] These diseases are characterized by a high accumulation of the pathogenic prion protein (scrapie form) [PrP^{Sc}] in the central nervous system. Although there is no epidemiologic evidence of transmission of a TSE through the administration of plasma-derived proteins,[9] the potential for transmission of CJD to humans remains a concern. Thus, manufacturers of therapeutic proteins have implemented in-process procedures to minimize the risk of the transmission of viruses and prions.

Viruses can be cleared from therapeutic proteins by inactivation (irreversible loss of viral infectivity using physical or chemical methods such as heat, low pH treatment, or solvent/detergent treatment) or removal (physical separation by precipitation, chromatography, or advanced filtration techniques).[9] In contrast, prions are very resistant to many methods used to inactivate viruses, including the use of heat, solvent detergents (S/D), and ionizing radiation. More extreme inactivation methods, such as caustic treatment and autoclaving, which inactivate and destroy the prion, can also destroy the biologic product itself.[9] Thus, the physical removal of prions through filtration is the only viable method to reduce the theoretical risk associated with the transmission of TSEs.

Viral Validation Studies

Guidelines set forth by the European Committee for Proprietary Medicinal Products (CPMP) recommend the per-

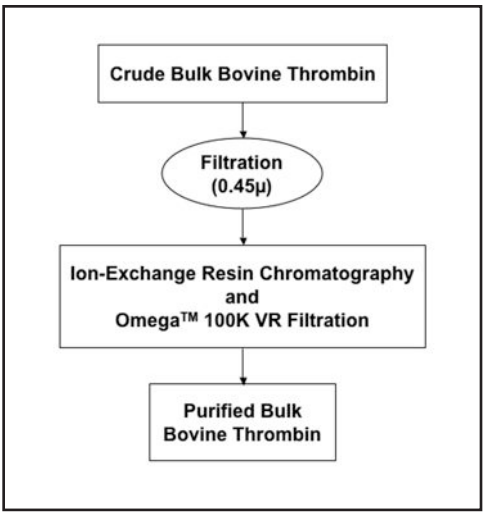

Figure 1. The bovine thrombin purification process.

formance of viral validation studies to quantitatively evaluate the ability of manufacturing processes to remove viral contaminants from biological products.[11] In the study results presented here, scaled-down models of the production process were used. Production samples were deliberately spiked with known amounts of virus, the spiked samples were put through the scaled-down manufacturing processes, and virus titer was determined in the pre- and post-filtration samples in order to calculate the log reduction factor for each virus considered.

Effective virus reduction is measured in two ways: degree of reduction and robustness of reduction. Virus reduction is expressed on a logarithmic scale. A virus clearance removal step that clears 4 $\log_{10}$ units or more of initial virus load (equivalent to a 10,000-fold reduction in titer) is considered effective.[9] A step that clears virus regardless of variability in production parameters and is reproducible on a larger scale is considered a robust step.[9,11] Viruses chosen for validation studies (model viruses) are those that most closely resemble probable viral contaminants for the target biological product and represent a wide range of physicochemical properties. The latter recommendation is designed to test the virus removal method's overall effectiveness in eliminating viruses. Thus, enveloped and non-enveloped viral contaminants should always be included in the panel of test-model viruses.

Viral Removal Filtration Systems

Viral removal filtration systems have been increasingly used to improve the safety profile of biologic agents.[12] The Omega 100K VR tangential-flow-filtration membrane filtration system (Pall Filtron, Northborough, MA) is one such system (Figure 1). The 100K model is designed to filter out proteins of 80-100 kiloDaltons (kDa) or less.[12] It does, however, retain very small viruses, as the Omega 100K VR system removes more than 4 logs of porcine parvovirus, which is a tiny (20-nm) non-enveloped virus.[12] This filtration method is ideal for removing viruses and large protein impurities by size exclusion from chromatographically purified bovine thrombin because other methods of viral clearance that apply heat, pH changes, and UV irradiation result in excessive thrombin degradation, and S/D inactivation is effective only on enveloped viruses.[9,12]

Recent data suggest that size-exclusion filtration techniques may be the most effective method for the removal of prions.[12] The TSE spiking agent used in prion clearance/validation studies is typically the hamster 263K strain scrapie (PrP^{Sc}).[9] The hamster 263K strain scrapie agent is considered a good model for the TSE pathogen for the following reasons: prions are well-conserved molecules with 89% amino acid sequence homology between hamster and other species, including humans,[13] and prions from various species share its antigenicity.[14] In addition, other physico-

Table 1. Characterization of Model Viruses Used in Virus Validation Studies

Virus	Genome	Enveloped	Family	Size (nm)	Resistance to Physicochemical Reagents
BPV	DNA	No	Parvo	20–25	High
XMuLV	RNA	Yes	Retro	80–100	Low
PRV	DNA	Yes	Herpes	150–200	Medium
BVDV	RNA	Yes	Flavi	40–70	Medium

BPV = bovine parvovirus; XMuLV = xenotropic murine leukemia virus; PRV = pseudorabies virus; BVDV = bovine viral diarrhea virus

chemical properties such as general resistance to proteinase K and prions from tissues of different species can be extracted using the same protocols.[14,15]

The purpose of this study was to evaluate the ability of the Omega l00K VR membrane filtration step used in the manufacture of bovine thrombin (Thrombin-JMI, King Pharmaceuticals, Inc.), to reduce or eliminate viruses and prions from chromatographically purified bovine thrombin.

METHODS

The studies were performed in compliance with the regulations of the US FDA's Good Laboratory Practices, the United Kingdom's Good Laboratory Practices, the Japanese Good Laboratory Practices standard, and the Organization for Economic Cooperation and Development's Principles of Good Laboratory Practice.

Thrombin was obtained through the conversion of prothrombin, extracted from bovine plasma in the presence of thromboplastin. Bovine plasma is obtained from herds that are inspected and comply with all current US Department of Agriculture and food industry standards.

The Omega 100K VR membrane filtration procedure consists of filtering 80% of the initial purified thrombin volume and diafiltering the remaining 20% of the volume with sodium chloride (NaCl) solution to maximize thrombin recovery. Virus and prion clearance by the Omega 100K VR filtration was evaluated using scaled-down models of the filtration procedure in which filtration conditions duplicate those conditions used during large scale production: feed and retentate pressure; ratio of purified thrombin to filter surface area, 400 mL/0.1 ft^2; potency of pre-filtration thrombin (Sample 4A), approximately 20,000 units/mL (virus clearance studies and run #1 of prion clearance studies). The integrity of the filter was confirmed before and after thrombin filtration. Virus and prion validation studies were performed by BioReliance (Rockville, MD).

Virus Clearance

Samples of a highly concentrated chromatographically purified thrombin solution were evaluated for viral clearance. Each sample had a potency of approximately 20,000 units/mL and a specific activity of ≥1500 units/mg of protein. The major component of this purified bovine thrombin solution was α-thrombin, which has a molecular weight of approximately 40 kDa.

In order to examine the clearance of potential and unexpected viral contaminants, a panel of four model viruses representing enveloped and nonenveloped

Table 2. Virus Removal by the Omega 100K VR Filtration Step of the Bovine Thrombin Manufacturing Process

	Log10 Reduction Factor ± SD			
	BPV	XMuLV	PRV	BVDV
Run #1	3.83 ± 0.40	3.86 ± 0.47	≥4.92 ± 0.16	4.35 ± 0.69
Run #2	3.62 ± 0.38	4.47 ± 0.60	≥4.92 ± 0.16	4.22 ± 0.64
Average log reduction factor	3.74 ± 0.39	4.26 ± 0.54	≥4.92 ± 0.16	4.29 ± 0.67

BPV = bovine parvovirus; XMuLV = xenotropic murine leukemia virus; PRV = pseudorabies virus; BVDV = bovine viral diarrhea virus

viruses and a broad range of physical and chemical characteristics were selected: bovine parvovirus (BPV), xenotropic murine leukemia virus (XMuLV), pseudorabies virus (PRV), and bovine viral diarrhea virus (BVDV) (Table 1).

Test samples (400 mL) of chromatographically purified bovine thrombin were spiked with high titers of one of the four model viruses. The volume of the virus spike was 20 mL per 400-mL bovine thrombin sample (5% vol/vol), consistent with Committee for Proprietary Medicinal Products (CPMP) guidelines (European Agency for the Evaluation of Medicinal Products). Spiked thrombin samples were passed through a 0.45-µ filter to remove any virus aggregates and then filtered through a 0.1-ft^2 Omega 100K VR membrane. When 340 mL of filtrate was collected, the remaining 80 mL of retentate solution was diafiltered with 6 times the retentate volume using a 0.65-M NaCl solution, producing a total permeate volume of about 820 mL. These filtration conditions have been shown to yield acceptable thrombin recovery as well as enhanced degree of thrombin purity.[16]

Two filtration runs were performed for each of the four viruses, one at a target feed pressure of 8 ± 2 pounds per square inch (psi) and one at a target feed pressure of 12 ± 2 psi. A new Omega 100K VR membrane was used for each run. Pre- and post-filtration samples were assayed for viruses using validated cell-based infectivity assays.

Stock viruses were prepared by infection of baby hamster kidney cells for PRV, infection of Madin Darby bovine kidney cells for BVD, and infection of chronically infected mink lung cells for XMuLV. For each virus, the cytotoxicity and potential interference with the infectivity assay were determined. The lowest sample dilution that could be evaluated without significant cytotoxicity or viral interference was 1:3 for PRV, 1:30 for XMuLV, 1:300 for BVDV, and 1:300 for BPV. These results were used to perform appropriate dilutions on the spiked thrombin load samples in order to eliminate any potential for cytotoxicity and viral interference with the assay.

Prion Clearance

Purified thrombin samples were spiked with lysolecithin-treated, sonicated, and filtered (through 0.45-µ, 0.22-µ, and 0.1-µ filters) scrapie brain homogenate from hamsters inoculated with the 263K strain of hamster-adapted scrapie. Each 400-mL of purified thrombin sample was spiked with 8 mL of scrapie agent (ratio, 1:50 vol/vol). Of each 408-mL spiked sample, 12 mL was reserved for pre-filtration quantitative analysis. The remainder of the spiked sample (396 mL) was filtered through a 0.1-ft^2 Pall Omega 100K VR membrane. The ratio

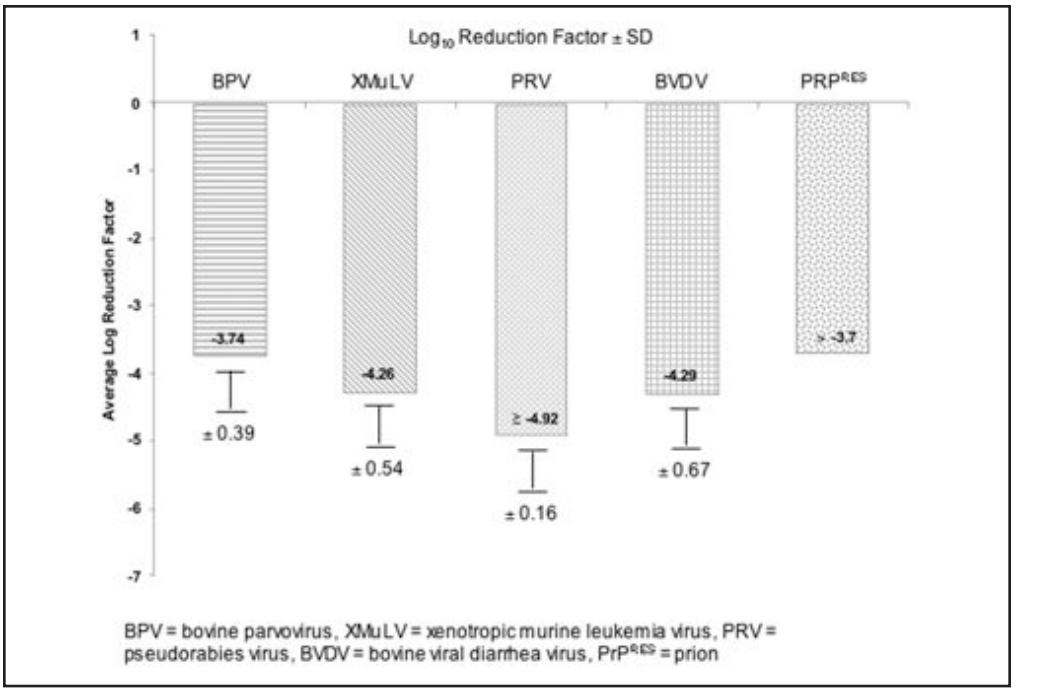

Figure 2. Virus and PrP^{RES} (prion) removal by the Omega 100K VR filtration step of the bovine thrombin manufacturing process.

of bovine thrombin volume to filter surface area was 4L/ft^2. When 315 mL of filtrate (ie, approximately 80% of the initial volume) was collected, the remaining sample was diafiltered with approximately 6 times the retentate volume (475 mL) using 0.65 M NaCl. The feed pressure was maintained at approximately 10 psi. Three filtration runs were performed on 3 separate scrapie-spiked thrombin samples. The pre- and postfiltration samples were stored at -60°C or below prior to performing the prion Western blot assay.

The pre- and post-filtration samples were evaluated by a validated Western blot assay with specificity for the protease-resistant form of the prion particle (PrP^{RES}), which is a surrogate marker for TSE infectivity.[17] Samples were prepared for Western blot assay as described by Lee et al.[17] The samples were first digested with proteinase K. A series of 0.5 $\log_{10}$ dilutions of the proteinase K–digested samples were electrophoretically separated on a Tris-glycine gel and transferred to a nitrocellulose membrane. The membrane was then incubated with a monoclonal antibody against PrP^{RES} (primary antibody), detected by chemiluminescence, then reincubated with an alkaline phosphatase-conjugated secondary antibody directed against the primary antibody. The membrane was then incubated with an alkaline phosphatase substrate (CDP-Star; New England BioLabs, Inc., Ipswich, MA) and a membrane enhancer (Nitro-Block-II; PerkinElmer, Waltham, MA) and exposed to Kodak BXAR-2 (VWR, West Chester, PA) films for signal detection. The presence of scrapie prion protein in a sample is indicated by detection of an approximately 30-KDa band. The positive control was 10% scrapie brain homogenate (hamster-adapted 263K strain; titer of 7.2 $\log_{10}$ PrP^{RES}/mL); the negative control was

Table 3. Removal of PrP^{RES} (Prion) by the Omega 100K VR Filtration Step of the Bovine Thrombin Manufacturing Process

	Log_{10} Reduction Factor
Run #1	3.6
Run #2	>3.7
Run #3	>3.7

0.1% bovine serum albumin in 1X Dulbecco's phosphate buffered saline (Sigma Chemical Company, St. Louis, MO). Western blot analyses were performed by BioReliance (Rockville, MD).

Scrapie agent interference assay was performed to evaluate the potential for interference of the test article with the detection of PrP^{RES} by Western blot analysis. The assay results indicated that the lowest dilution that could be evaluated by Western blot analysis without significant interference was "undiluted" for the Omega 100K VR filtration load test article.

Calculation of Virus/Prion Reduction Factor

Clearance of virus/prion was assessed by calculation of the log_{10} reduction factor (RF) as follows[11]:

RF = log10 ((V1 x T1)/(V2 x T2)).
[V1 = volume of starting (input) material, T1 = virus/prion concentration in starting material; V2 = volume of material after filtration step; T2 = concentration of virus/prion after filtration step.]

RESULTS

Scale-Down Validation

The experimental filtration conditions used in the scaled-down production model were confirmed to be similar to the conditions used during large-scale production in terms of feed pressure, retentate pressure, cross flow, and throughput (volume of pre-filtration thrombin per ft^2 of Omega 100K VR membrane).

Viral Clearance

Across the 8 runs, the total filtration time ranged from 236 to 257 minutes for run #1 and from 190 to 223 minutes for run #2. The feed pressure was maintained at 8 to 10 psi for run #1 and at 12–14 psi for run #2. The retentate pressure was 0 ± 2 psi for all runs. At the beginning of run #1 and run #2, the cross flows ranged from 41 to 44 mL/min and from 54 to 56 mL/min, respectively. The ratio of thrombin volume to filter surface area was 400 mL/0.1 ft^2.

The titer of the spiking virus control for BPV was equal to 7.35 $TCID_{50}$/mL (log median tissue culture infective dose 50% per mL). The titers for the other spiking viruses were 7.38, 8.30, and 7.70 for PRV, BVDV, and XMuLV respectively.

Filtration under targeted manufacturing conditions resulted in an average ≥4.92 for PRV, 4.26 for XMuLV, 4.29 for BVDV, and 3.74 for BPV (Table 2, Figure 2).

Prion Clearance

The potencies of the pre-filtration thrombin sample were 20,000 units/mL (run #1) and 50,286 units/mL (run #2 and run #3). For runs #1–3, respectively, the final postfiltration thrombin volumes were 790 mL, 800 mL, and 800 mL; the total filtration times were 172, 174, and 155 minutes; and the feed pressures were 10, 8, and 12 psi. The retentate pressure was 0 (all runs). The ratio of thrombin to filter surface area was 400 mL/0.1 ft^2.

In each Western blot assay performed in this study, the controls met the criteria for assay validity. The positive control titer demonstrated the approximately 30-KDa band and was within ± 1.0 log of the predetermined titer, and the negative control showed

no evidence of an immunoreactive (30-KDa) band.

The log reduction factors for PrP^{RES} were 3.6, >3.7, and >3.7 for runs #1–3, respectively (Table 3). The average log reduction factor for PrP^{RES} was >3.7 (Figure 2).

DISCUSSION

Validation of the purification procedures for removing pathogens in biological therapeutic agents is essential in establishing the safety of these products. While the purification of therapeutic proteins by chromatography may coincidentally remove viruses, size exclusion filtration methods, such as that used for the manufacture of bovine thrombin, are designed specifically for the removal of viruses and operate under defined conditions (eg, filtration pressure, flow rate, protein load/filter area ratio) that are known to effectively remove viruses.

Size exclusion filtration is often the purification method of choice for virus removal. It is nondestructive of biological products, capable of removing high titers of both enveloped and nonenveloped viruses, and does not induce biologic or immunologic reactions.[9,12] The pore size of the membrane relative to the size of viral contaminants determines the overall extent of virus removal. For example, the Omega 100K VR membrane filter removes more than 4 log of porcine parvovirus (PPV), a very small (20-nm) nonenveloped virus that is extremely resistant to physicochemical treatments.[18] This filtration process preserves the integrity of the end product, bovine thrombin, and enhances the purity of chromatographically partitioned bovine thrombin.[16]

In the case of bovine thrombin, size exclusion filtration is ideal for removing viruses because other methods of virus inactivation such as heat, pH, and ultraviolet irradiations result in excessive thrombin degradation,[16] and S/D inactivation is effective only on enveloped viruses.[9] The Omega 100K VR membrane filter is used at the end of the production process, prior to aseptic filtration and filling, thereby eliminating the risk of recontamination by viruses and other infectious pathogens.

The results of the current virus and prion validation studies demonstrate that the Omega 100K VR membrane filtration step in the manufacturing process of bovine thrombin effectively reduces both viruses and TSE agents (prions). The filtration step was effective in removing all four viruses from the chromatographically purified bovine thrombin samples (Sample 4A). The high $\log_{10}$ reduction factors achieved by filtration and the similarity of the results between the two runs for each virus suggest the robustness of the filtration step and the validity of the results.[9] The average $\log_{10}$ reduction factors exceeded 4 for all of the test viruses except BPV, for which the average $\log_{10}$ reduction factor was 3.74 ± 0.39. However, the failure to demonstrate 4 logs of reduction for BPV is likely attributable to the experimental conditions, specifically the moderate cytotoxicity of BPV, which necessitated the dilution of BPV samples to 1:300 (vol/vol) prior to titration in order to eliminate cytotoxicity/viral interference issues. This limited the achievement of high titer results for the load samples. This point can be illustrated using the value for the titer of the spiking virus control (7.35 $TCID_{50}$/mL). Spiking the thrombin sample in a ratio of 1:20 (20 mL of virus to 400 mL of thrombin) would dilute the titer of the load solution downward to a theoretical value of 6.05 $TCID_{50}$/mL [7.35 – log(20) = 7.35 – 1.3]. After an additional dilution of 1:300 to eliminate cytotoxicity and viral interference with the assay, the titer would be further reduced by log(300) (= 2.48) to a theoretical titer of 3.57 (6.05 – 2.48), making it impossible to achieve a 4 log

reduction. A similar situation existed for BVDV, for which dilutions higher than 1:300 could not be used. As a result, it is likely that the final log reduction factors were underestimated for both BPV and BVDV. Overall, the results of the viral validation studies are consistent with the CPMP guidelines of the European Agency for the Evaluation of Medicinal Products,[11] in which $\log_{10}$ reductions of 4 indicate a clear effect of the purification method—in this case, size exclusion filtration.

This study also demonstrated that the Omega 100K VR filtration process is also capable of removing a significant amount of potential prion contaminants from chromatographically purified thrombin. After Omega 100K VR filtration, the experimental prion (PrP^{RES}) level in bovine thrombin was reduced by 3.6 logs or more. Reductions in PrP^{RES} demonstrated by the sensitive and specific Western blot assay method used in the current study[17] correlate closely with reductions in TSE infectivity, as shown by Lee and colleagues.[19] Thus, the Western blot assay results indicate that the filtration process utilized in the production of bovine thrombin is capable of significantly reducing TSE infectivity potential in the final product.

CONCLUSION

In conclusion, validation of the Omega 100K VR membrane filtration process in the manufacture of bovine thrombin using a panel of four model viruses and a surrogate marker for TSE (PrP^{RES}) demonstrated effective removal of viruses and prions. The effectiveness of this filtration system against viruses with a wide range of characteristics, including size, type of nucleic acid, resistance to heat and chemicals, and presence/absence of lipid envelope, suggest that this purification step in the manufacture of bovine thrombin removes unknown viral pathogens. The utilization of the filtration step in the manufacturing process further improves the biologic safety of bovine thrombin while maintaining its integrity as a rapidly effective active hemostat for surgical procedures. It has also been demonstrated to be robust, since viral clearance occurs regardless of variability in production parameters and is reproducible on a larger scale.

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