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Removal and Inactivation of Viruses during Manufacture of a High Purity Antihemophilic Factor VIII Concentrate from Human Plasma

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Abstract The purpose of this study was to examine the efficacy and mechanism of the cryo-precipitation, solvent/detergent (S/D) treatment, monoclonal anti-FVIIIc antibody (mAb) column chromatography, Q-Sepharose column chromatography, and lyophilization involved in the manufacture of antihemophilic factor VIII (GreenMono) from human plasma, in the removal and/or inactivation of blood-borne viruses. A variety of experimental model viruses for human pathogenic viruses, including the bovine viral diarrhoea virus (BVDV), bovine herpes virus (BHV), murine encephalomyocarditis virus (EMCV), and porcine parvovirus (PPV), were all selected for this study. BHV and EMCV were effectively partitioned from a factor VIII during the cryo-precipitation with a log reduction factor of 2.83 and 3.24, respectively. S/D treatment using the organic solvent, tri(n-butyl) phosphate (TNBP), and the detergent, Triton X-100, was a robust and effective step in inactivating enveloped viruses. The titers of BHV and BVDV were reduced from the initial titer of 8.85 and 7.89 $\log_{10}$ $TCID_{50}$, respectively, reaching undetectable levels within 1 min of the S/D treatment. The mAb chromatography was the most effective step for removing nonenveloped viruses, EMCV and PPV, with the log reduction factors of 4.86 and 3.72, respectively. Q-Sepharose chromatography showed a significant efficacy for partitioning BHV, BVDV, EMCV, and PPV with the log reduction factors of 2.32, 2.49, 2.60, and 1.33, respectively. Lyophilization was an effective step in inactivatin g nonenveloped viruses rather than enveloped viruses, where the log reduction factors of BHV, BVDV, EMCV, and PPV were 1.41, 1.79, 4.76, and 2.05, respectively. The cumulative log reduction factors of BHV, BVDV, EMCV, and PPV were ≥11.12, ≥7.88, 15.46, and 7.10, respectively. These results indicate that the production process for GreenMono has a sufficient virus-reducing capacity to achieve a high margin of the virus safety.

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Hemophilia A is an inherited bleeding disorder, in which the blood clotting protein factor VIII is deficient or abnormal [10]. Until now, hemophilia has generally been treated by injecting with coagulation factor concentrates, which are made from pooled plasma of many blood donors [1]. Therefore, special precaution must be taken during the production of these proteins to exclude the possibility of transmitting infectious diseases to the recipients by these products [7, 19]. The major blood-borne viruses of clinical concern include the human immunodeficiency virus (HIV), human T-cell leukemia virus (HTLV), hepatitis A, B, C, and G viruses, and parvoviruses [3, 4, 20, 23-26].

Methods to improve the safety of plasma-derived products are based on four principles: a) careful selection of donors; b) careful screening of donated plasma for known infectious agents; c) the use of validated manufacturing methods, which include specific steps designed to remove or inactivate viruses; and d) quality control of final products. Implementation of multiple viral clearance steps or new technologies has been highly recommended for plasma-derivative manufacturers [11, 14].

The concept of affinity chromatography utilizing monoclonal antibody (mAb) ligands, with a capacity to bind only to the specific coagulation protein, was developed in the 1980s. It resulted in the introduction of coagulation products of a very high degree of purity. Green Cross Plasma Derivatives Co. is currently producing a high purity antihemophilic factor VIII (GreenMono) using a monoclonal anti-FVIIIc immunoaffinity chromatography. This process includes another chromatography step using a Q-Sepharose anion exchange column. These chromatographic procedures may be powerful means by which blood-borne viruses can be physically separated from the factor VIII. This process also adopts a

solvent/detergent (S/D) virus inactivation method which has been proven to be extremely effective for lipid-enveloped viruses [15].

Establishing freedom of a biopharmaceutical product from infectious viruses, in many instances, will not derive solely from direct testing for their presence, but also from a demonstration that the manufacturing process is capable of removing or inactivating the virus. Validation of the process for viral inactivation and/or removal can play an essential and important role in establishing the safety of biological products, especially when there is a high potential for the source material to be contaminated with a virus that is known to be pathogenic for mankind. Also, since many instances of contamination in the past have occurred with agents whose presence was not known or even suspected at the time of the manufacturing procedure, an evaluation of the process would provide a measure of confidence in which a wide range of viruses, including unknown and harmful viruses, may be eliminated [11]. In this regard, we have carried out a process validation study to evaluate the efficacy of individual steps for removal and/or inactivation of several model viruses during the manufacturing process of GreenMono.

MATERIALS AND METHODS

Viruses and Cells

The viruses used in this study were selected mostly to demonstrate the inactivation and/or removal of viruses with a range of biophysical and structural features which may reflect the presence of any unknown or unidentified contaminants in the starting material and display a significant resistance to physical or chemical agents [11, 14]. The viruses selected based on this rationale were as follows:

Bovine herpes virus (BHV) - enveloped, large, double-stranded DNA virus (ATCC VR-188) [17]. Herpesviruses can remain as latent infections within lymphoid cells, and several herpesviruses, such as HSV-1, HSV-2, HCMV, HHV-6, HHV-7, and HHV-8 are potentially transmissible by blood and plasma. Therefore, BHV was selected since it is representative of this class of virus, can be obtained in a sufficient titer for validation studies, and is not neutralized by the presence of antibodies in the plasma. For the propagation and titration of BHV, Madin-Derby bovine kidney (MDBK) cells (ATCC CRL-22) were used, which were grown in a high glucose Dulbecco's modified Eagle's medium (HG DMEM) containing 2% fetal bovine serum (FBS) plus nonessential amino acids (NEAA).

Bovine viral diarrhoea virus (BVDV) - enveloped, medium-sized, single-stranded RNA virus (ATCC VR-534) with a medium resistance to physicochemical reagents [17]. BVDV belongs to the Flaviviridae family, which also includes the hepatitis C virus [6, 21]. BVDV is therefore a suitable model virus where hepatitis C is of concern, particularly in products derived from human blood. It is also a model for the hepatitis G virus. For the propagation and titration of BVDV, bovine turbinate (BT) cells (ATCC CRL-1390) were used which were grown in HG DMEM containing 2% FBS plus NEAA.

Murine encephalomyocarditis virus (EMCV) - nonenveloped, small, single-stranded RNA virus (ATCC VR-129B) with a medium to high resistance to physicochemical reagents [17]. EMCV provides a severe test for the validation for the removal and/or inactivation of viruses. EMCV belongs to the Picornaviridae family, which also includes the hepatitis A virus [13]. For the propagation and titration of EMCV, Vero cells (ATCC CCL-81) were used which were grown in HG DMEM containing 2% FBS plus NEAA.

Porcine parvovirus (PPV) - nonenveloped, small, single-stranded DNA virus (ATCC VR-742) [17, 27]. This virus has a high resistance to a range of physicochemical reagents and is a known contaminant of porcine blood. Therefore, it provides an intense test for the validation of a process for removal and/or inactivation of viruses. For the propagation and titration of PPV, minipig kidney (MPK) cells (ATCC CCL-166) were used which were grown in HG DMEM containing 2% FBS.

Titration of Viruses

An aliquot from each sample and an appropriate control were titrated immediately after collection, in 7-fold serial dilutions to the end point, using a quantal 50% tissue culture infectious dose ($TCID_{50}$) assay [12]. Indicator cell monolayers in 24-well culture plates were then infected using at least eight replicates of 0.25 ml of the appropriate dilution of each sample or the positive control. Negative control wells were mock-infected using at least eight replicates of 0.25 ml of the culture medium. The plates were then incubated at 35°C for approximately 1 h, and the wells were fed with 1 ml of the tissue culture medium.

As a part of the virus validation protocol, cytotoxicity, interference, and load titer tests were also performed. The cytotoxicity tests were performed on those samples generated for virus titration in the virus-spiking experiments to control any possible cytotoxic effects on the indicator cells which would interfere with the virus titration. Indicator cell monolayers in 24-well culture plates were infected using at least eight replicates of 0.25 ml of a range of doubling dilutions of each sample. The plates were then incubated at 35°C for approximately 1 h, and the wells were fed with 1 ml of the tissue culture medium. After appropriate incubation, the cytotoxic effect of each sample was measured. The interference tests were performed to determine whether the starting materials for the virus-spiking studies exerted an inhibitory effect on the ability of the cell lines to permit the detection of the virus. Aliquots of virus were spiked into

doubling dilutions of sample to give final concentrations within the range of 10^0 to 10^3 infectious units. The dilutions were then assayed for the presence of infectious virus with two replicates per virus concentration per buffer dilution tested. The load titer assays were performed to determine precisely the point at which spiking the virus into the starting material resulted in a loss in the virus titer.

Virus-Spiking Studies

As shown in Fig. 1, the GreenMono manufacturing process includes cryo-precipitation, S/D treatment, column chromatography using mAb and Q-Sepharose, and lyophilization steps. Process validation experiments were carried out by spiking the starting material for each step of the process with an aliquot of the virus stock solution in a volume that was 10% (v/v) of the total volume of the material as described in the previous reports [14, 15]. This was to ensure that the nature of the starting material was not affected by adding virus in the tissue culture medium. A control sample was taken after adding virus to the starting material. After subjecting the sample to the processing step, appropriate fractions were collected for evaluating infectious virus.

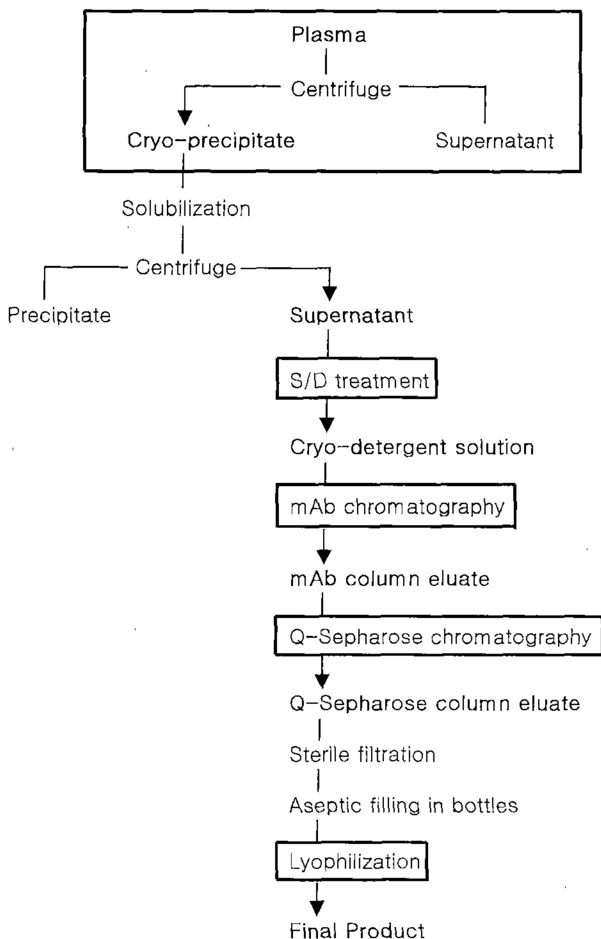

Fig. 1. Flow diagram of manufacturing process for GreenMono.
The boxes indicate the validation steps evaluated for virus removal and/or inactivation.

The first step in factor VIII manufacture is to separate the cryo-precipitate from other plasma proteins. To determine how virus partitions during this process, 5 ml of virus stock was spiked to 45 ml of three different pooled plasma samples which had been thawed at 4°C. The cryo-precipitate was collected by slow speed centrifugation (4,000 ×g, 3 min), and the virus content of the supernatant and resuspended cryo-precipitate fractions were compared with that of the spiked-pooled plasma.

Five ml of the virus stock was spiked to 45 ml of Pre-S/D solution (supernatants of solubilized cryo-precipitate) equilibrated at 22°C. The virus-spiked solution was treated with TNBP and Triton X-100, with the final concentration of 0.3% (v/v) and 1.0% (v/v), respectively, and then incubated at 22°C. Samples were taken at the indicated time periods, and then they were diluted 250-fold with a tissue culture medium to prevent further inactivation of the viruses and cytotoxicity of the S/D treated solution to the indicator cells. To further assess the robustness of S/D treatment, the virus-spiked solution was treated with 50% and 25% of the specified S/D concentrations.

The partitioning profile of viruses during mAb chromatography was assessed in a validated scale-down process. The monoclonal anti-FVIIIc antibodies were supplied by Hyland Division, Baxter Healthcare Corp. Table 1 shows a typical comparison of some process parameters for the manufacturing and scale-down processes of mAb column chromatography. The cryo-detergent solution was spiked with a virus and then a sample was immediately removed for virus titration. The remaining material was applied to the mAb chromatography column to which a factor VIII specifically binds. The mAb column was extensively washed to remove unbound protein contaminants, virus, and S/D mixture. After washing the column with 0.05 M imidazole buffer containing 5% ethylene glycol (pH 6.4), factor VIII was eluted with buffer containing 0.05 M imidazole and 40% ethylene glycol (pH 6.5). After eluting factor VIII, the column was washed with a high salt buffer containing 2.0 M of sodium chloride to show how much the virus still remained bound to the column. The unbound, high salt wash fractions were collected and an aliquot of each sample was immediately titrated.

To evaluate the effectiveness of the Q-Sepharose column chromatography step in eliminating viruses, the elution profile of the virus during the Q-Sepharose chromatography was assessed as described previously [15].

The final step in manufacturing factor VIII was freeze-drying. The virus stock was spiked to the final factor VIII concentrate solution and then the virus-spiked samples were distributed in final containers. The titer of viruses was measured before and after the process of lyophilization using an experimental freeze dryer (VirTis, Genesis 25XL, U.S.A.).

Table 1. Comparison of some typical process parameters for the manufacturing and scale-down processes of mAb column chromatography.

Item	Manufacturing process	Scale-down process	Scale-down factor*
Resin-bed dimensions	25 cm diameter 9.4 cm height	1 cm diameter 9.4 cm height	623
Volume of mAb gel	4,612 ml	7.4 ml	623
Volume of the cryo-detergent solution (starting material)	187 l	0.3 l	623
Flow rate of elution	155.8 ml/min	0.25 ml/min	623
Average protein concentration of eluate after chromatography	8.71±0.17 mg/ml	8.68±0.02 mg/ml	-

*Scale-down factor was calculated by dividing the parameter of manufacturing process by that of scale-down process.

Calculation of Virus Reduction Factors

The virus reduction factor for an individual purification or inactivation step was defined as the $\log_{10}$ of the ratio of the virus load in the spiked starting material divided by the virus load in the post-process material [11, 14]. The formula takes into account both the titers and volumes of the materials before and after the processing step.

$$10^{Ri}=(v^{I})(10^{aI})/(v^{II})(10^{aII})$$

where: Ri = reduction factor for a given stage, v^I = volume of the input material, aI = titer of the virus in the input material, v^{II} = volume of the retained output material, aII = titer of the virus in the output material

All the experiments were carried out three times and mean values of three independent experiments were evaluated.

RESULTS

Partitioning of Viruses during Cryo-Precipitation

The partitioning profile of BHV and EMCV after cryo-precipitation was assessed in order to evaluate the efficacy of the process in eliminating these viruses (Table 2). Most of the infectious viruses were detected in the supernatant fractions, while only a few viruses were present in the cryo-precipitate, indicating that BHV and EMCV were effectively partitioned from factor VIII. The log reduction factors achieved were 2.83 for BHV and 3.24 for EMCV.

Inactivation of Enveloped Viruses during S/D Treatment

The effectiveness of S/D treatment in inactivating enveloped viruses was determined (Table 3). BHV and BVDV were inactivated to undetectable levels within the first min of the total 1 h treatment time. The log reduction factors achieved for BHV and BVDV were ≥4.56 and ≥3.60, respectively.

To further assess the robustness of S/D treatment, the kinetics of inactivation of BHV and BVDV were studied as the concentration of TNBP and Triton X-100 decreased (Table 4). Virus-spiked solutions were treated with the concentrations of 50% and 25% of the specified S/D concentrations (0.3% TNBP and 1.0% Triton X-100), and then the results were compared with that obtained with the specified S/D concentrations. S/D treatment with the reduced concentrations was also very effective in inactivating BHV and BVDV. BHV inactivation was complete within 5 min even when the concentrations of TNBP and Triton X-100 were reduced to 25% of the specified concentrations. Also, BVDV was completely inactivated within 30 min of incubation with the concentration level of 25% in terms of the specified S/D concentrations.

Removal of Nonenveloped Viruses during mAb Column Chromatography

The partitioning profile of EMCV and PPV during mAb column chromatography was assessed in order to evaluate the efficacy of the process in eliminating these nonenveloped viruses (Table 5). The mAb column chromatography was an effective step to remove EMCV and PPV with the log reduction factors of 4.86 and 3.72, respectively. Most of

Table 2. Partitioning of BHV and EMCV during cryo-precipitation.

Sample	Total virus titer ($\log_{10}$ $TCID_{50}$)	
	BHV	EMCV
Plasma spiked with virus	9.59±0.16	8.78±0.11
Cryo-precipitate	6.76±0.11	5.54±0.06
Supernatant fraction	8.80±0.23	7.56±0.06
Log reduction factor	2.83	3.24

Table 3. Inactivation of BHV and BVDV during S/D treatment.

Sample	Total virus titer ($\log_{10}$ $TCID_{50}$)	
	BHV	BVDV
Starting material spiked with virus	8.85±0.22	7.89±0.06
1 min after S/D treatment	≤4.29*	≤4.29
5 min after S/D treatment	≤4.29	≤4.29
30 min after S/D treatment	≤4.29	≤4.29
60 min after S/D treatment	≤4.29	≤4.29
Log reduction factor	≥4.56	≥3.60

*No infectious virus was detected. These values were calculated using a theoretical minimal detection level of infectious virus with a 98% confidence level.

Table 4. Effect of reduced levels of TNBP and Triton X-100 on inactivation of BHV and BVDV.

S/D Concentration (%)		Total virus titer (Log_{10} $TCID_{50}$)				
		BHV			BVDV	
TNBP	Triton X-100	Initial	5 min	30 min	Initial	30 min
0.30	1.0	8.85±0.22	ND*	ND	7.89±0.06	ND
0.15	0.5	8.76±0.25	ND	ND	7.78±0.12	ND
0.075	0.25	8.94±0.46	ND	ND	7.92±0.24	ND

*No infectious virus was detected.

Table 5. Removal of EMCV and PPV during mAb column chromatography.

Sample	Total virus titer (Log_{10} $TCID_{50}$)	
	EMCV	PPV
Cryo-detergent solution spiked with viruses	9.67±0.18	7.85±0.06
Unbound fraction	9.30±0.16	6.97±0.06
Wash fraction	6.72±0.06	5.55±0.13
Eluate fraction	4.81±0.13	4.13±0.16
High salt wash fraction	3.12±0.08	3.02±0.14
Log reduction factor	4.86	3.72

the infectious viruses were present in the unbound and wash fractions.

Removal of Viruses during Q-Sepharose Column Chromatography

The elution profile of viruses during Q-Sepharose column chromatography was assessed in order to evaluate the efficacy of the process in eliminating both enveloped and nonenveloped viruses (Table 6). The results indicate that the Q-Sepharose column was moderately effective for removing all the viruses tested. The log reduction factors achieved were 2.32 for BHV, 2.49 for BVDV, 2.60 for EMCV, and 1.33 for PPV.

Inactivation of Viruses during Lyophilization

The final step in manufacturing the highly purified factor VIII was freeze-drying. The effect of lyophilization on the inactivation of viruses was evaluated (Table 7). The results indicate that lyophilization was effective in inactivating all the viruses tested. Nonenveloped viruses were more sensitive to freeze-drying than enveloped viruses. The log reduction factors achieved were 1.41 for BHV, 1.79 for BVDV, 4.76 for EMCV, and 2.05 for PPV.

DISCUSSION

This study was conducted to establish the capacity and efficiency of the process to remove and/or inactivate viruses using a validated down-scale for manufacturing antihemophilic factor VIII (GreenMono) from human plasma. This kind of

Table 6. Removal of BHV, BVDV, EMCV, and PPV during Q-Sepharose column chromatography.

Sample	Total virus titer (Log_{10} $TCID_{50}$)			
	BHV	BVDV	EMCV	PPV
mAb column eluate spiked with virus	9.11±0.16	6.21±0.50	9.11±0.06	8.15±0.06
Unbound fraction	4.88±0.38	4.72±0.48	8.12±0.11	5.13±0.06
Post-rinse fraction	4.16±0.12	4.53±0.52	7.71±0.06	5.03±0.06
Wash fraction	2.99±0.32	4.06±0.46	7.22±0.21	4.02±0.06
Eluate fraction	6.79±0.16	3.72±0.62	6.51±0.11	6.82±0.02
High salt wash fraction	7.83±0.28	4.65±0.32	4.24±0.16	5.98±0.24
Log reduction factor	2.32	2.49	2.60	1.33

Table 7. Inactivation of viruses during lyophilization.

Sample	Total virus titer (Log_{10} $TCID_{50}$)			
	BHV	BVDV	EMCV	PPV
Before lyophilization	8.35±0.43	5.32±0.40	7.71±0.37	6.54±0.43
After lyophilization	6.94±0.48	3.53±0.42	2.95±0.36	4.49±0.34
Log reduction factor	1.41	1.79	4.76	2.05

process validation study is a key component in assessing the safety of a biopharmaceutical or pharmaceutical product derived from biological fluids, tissues, or organs of human or animal origin. It is therefore important that manufacturers design the purification process not only to consider the yield and purity of the product but also to include steps that will inactivate or remove viruses and other infectious agents. In this regard, the GreenMono manufacturing process has sufficient virus reducing steps which include cryo-precipitation, S/D treatment, column chromatography using mAb and Q-Sepharose, and lyophilization.

A great majority of BHV and EMCV did not cryo-precipitate with the factor VIII concentrate as indicated by the log reduction factors of 2.83 and 3.24, respectively, in Table 2. The cryo-precipitation process has also been reported to be highly effective in partitioning hepatitis A virus, with a log reduction factor of 2.63 [16].

The principal method for inactivating enveloped virus contamination in plasma products is the S/D treatment [8, 9, 18, 22]. The solvent frequently used in the S/D method is TNBP and the detergent is either Tween 80, Triton X-100, or sodium cholate. Organic solvent/detergent mixtures disrupt membranes of viruses which have lipid envelopes. The result is either complete structural disruption or destruction of the cell receptor recognition site. In both cases, the viruses are rendered noninfectious. The results of inactivation kinetics obtained from the present study showed that S/D treatment is a robust and effective step in eliminating enveloped viruses. Since viral load was reduced to undetectable levels within 1 min of the total 1 h period of incubation (Table 3), it could be further concluded that the extent of viral inactivation was independent of the initial viral load. Furthermore, BHV and BVDV were completely inactivated with the concentration of 25% of the specified S/D concentrations within 30 min of incubation, which was chosen as the worst case in the manufacturing process (Table 4). These results demonstrate the robustness of the S/D step in the manufacturing process of GreenMono in eliminating representative enveloped viruses. Previous report also showed that the titer of human immunodeficiency virus was reduced from an initial titer of 8.3 $\log_{10}$ $TCID_{50}$ to undetectable levels within 1 min of the S/D treatment [15].

The immunoaffinity column chromatography using anti-human factor VIII:C monoclonal antibody matrices was the most effective step for removing nonenveloped viruses from the factor VIII concentrates (Table 5). During the mAb column chromatography process, factor VIII specifically bound to the monoclonal antibodies, while the contaminants including viruses, S/D, and residual proteins were removed by extensive washing. In particular, most of the viruses did not bind to the mAb column, but flowed through the column. The mAb chromatographic step for GreenMono production might also provide powerful means to physically separated enveloped viruses from factor VIII. However, lipid-enveloped virus removal was not tested in this step, because the process material used in this step contains S/D. In other words, the presence of S/D would effectively inactivate any spiked enveloped virus, and thus the effectiveness of the mAb chromatography in eliminating these viruses can not be accurately explained.

The Q-Sepharose column chromatography, described as an ion-exchange step in the manufacture of factor VIII concentrates to further reduce impurities, was found to be effective against the test viruses with an exception of PPV (Table 6). The log reduction factor for PPV was 1.33, while those for BHV, BVDV, and EMCV were 2.32, 2.49, and 2.60, respectively. Previous report showed that HIV-1 was also successfully partitioned from factor VIII during the Q-Sepharose column chromatography with a log reduction factor of 4.1 [15]. Although most of the viruses were partitioned from factor VIII, infectivity of viruses was still detected in the high salt washing fraction, which indicates that extensive washing and cleaning of the Q-Sepharose column and validation of these processes are necessary to regenerate the column.

Freeze-drying is a standard method for stabilizing labile products with limited shelf-lives in a dilute solution. It has been known that virus infectivity has diminished substantially by going through lyophilization [2, 5]. The results obtained from this study indicate that lyophilization was effective in inactivating all the viruses tested. Nonenveloped viruses were more sensitive to freeze-drying than enveloped viruses (Table 7). The mechanism of lyophilization by which viruses are inactivated is poorly understood, but it might be due to the destabilization of quaternary interactions between components of the capsid. Under normal conditions, water of solvation will be important in maintaining a protein secondary structure and it may also be involved in a hydrogen bond formation between different subunits. This water would be removed during the lyophilization process, which could result in disruption of normal protein interactions.

The cumulative virus reduction factor for a manufacturing process is determined from the sum of the individual virus reduction factors based on individual process steps from different physicochemical methods [11]. The cumulative virus reduction factors achieved for different viruses by the process steps evaluated are presented in Table 8. The cumulative log reduction factors, ≥11.12 for BHV, ≥7.88 for BVDV, 15.46 for EMCV, and 7.10 for PPV, represented several magnitudes greater than the potential virus load of current plasma pools. Accordingly, these results indicate that the process steps in manufacturing GreenMono are capable of eliminating/inactivating a wide range of viruses which represent a broad spectrum of physicochemical attributes. Since the mechanism of virus elimination/inactivation in each step is different, the overall process of GreenMono production is robust in reducing the virus load.

Table 8. Virus log reduction factors achieved during production of GreenMono.

Process step	Lipid-enveloped virus		Nonenveloped virus	
	BHV	BVDV	EMCV	PPV
Cryo-precipitation	2.83	-*	3.24	-
S/D treatment	≥4.56	≥3.60	-	-
mAb chromatography	-	-	4.86	3.72
Q-Sepharose chromatography	2.32	2.49	2.60	1.33
Lyophilization	1.41	1.79	4.76	2.05
Cumulative log reduction factor	≥11.12	≥7.88	15.46	7.10

*Not determined.

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