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Improvement of Virus Safety of an Antihemophilc Factor IX by Virus Filtration Process

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Viral safety is an important prerequisite for clinical preparations of plasma-derived pharmaceuticals. One potential way to increase the safety of therapeutic biological products is the use of a virus-retentive filter. In order to increase the viral safety of human antihemophilic factor IX, particularly in regard to non-enveloped viruses, a virus removal process using a polyvinylidene fluoride membrane filter (Viresolve NFP) has been optimized. The most critical factor affecting the filtration efficiency was operating pH and the optimum pH was 6 or 7. Flow rate increased with increasing operating pressure and temperature. Recovery yield in the optimized production-scale process was 96%. No substantial changes were observed in the physical and biochemical characteristics of the filtered factor IX in comparison with those before filtration. A 47-mm disk membrane filter was used to simulate the process performance of the production-scale cartridges and to test if it could remove several experimental model viruses for human pathogenic viruses, including human hepatitis A virus (HAV), porcine parvovirus (PPV), murine encephalomyocarditis virus (EMCV), human immunodeficiency virus type 1 (HIV), bovine viral diarrhea virus (BVDV), and bovine herpes virus (BHV). Non-enveloped viruses (HAV, PPV, and EMCV) as well as enveloped viruses (HIV, BVDV, and BHV) were completely removed during filtration. The log reduction factors achieved were ≥6.12 for HAV, ≥4.28 for PPV, ≥5.33 for EMCV, ≥5.51 for HIV, ≥5.17 for BVDV, and ≥5.75 for BHV. These results indicate that the virus filtration process successfully improved the viral safety of factor IX.

Keywords: Antihemophilic factor IX, virus filter, virus removal, log reduction factor

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Hemophilia B is an inherited bleeding disorder, where the blood clotting factor IX is deficient or abnormal. Until now, hemophilia has generally been treated by the injection of coagulation factor concentrates made from the pooled plasma of many blood donors [28]. The use of biopharmaceutical products derived from human plasma has previously been associated with the frequent transmission of human immunodeficiency virus type 1 (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) [5, 16, 34]. The development of virus inactivation and/or removal technologies has greatly reduced the frequency of such transmissions [13, 20, 27]. Since the advent of solvent/detergent (S/D) treatment for plasma-derived products, there has been no reported transmission of enveloped viruses such as HIV, HBV, or HCV by treated products [37]. Although S/D-treated blood products have become widely available over the last 15 years, this treatment generally has no effect on non-enveloped viruses [31]. Consequently, the blood industry is paying particular attention to systems capable of removing and/or inactivating non-enveloped viruses, such as hepatitis A virus (HAV) and human parvovirus B19 (B19) [7, 9, 22, 26, 30, 33].

Recently, there have been a few reports describing HAV or B19 infections in hemophilic patients having received antihemophilic factor IX or VIII concentrate prepared from large plasma pools [10, 23, 32]. Therefore, the international regulation for the validation of HAV and B19 safety has had to be re-inforced.

One potential way to increase the safety of therapeutic biological products is the use of virus-retentive filters. Virus removal with membrane filtration has become a complementary and robust method for viral clearance, because it does not compromise the biological integrity of the product; additionally, when viral particle removal is based on size exclusion, it constitutes a robust mechanism for virus removal, as it is not affected by minor alteration in process parameters [2, 8]. Although there have been several

reports about the application of virus filters for removing possible contaminating viruses from biopharmaceuticals [3, 4, 11, 29, 38], less has been reported about the implementation of virus filter of pore-size 20 nm to particularly remove HAV and B19 from factor IX.

The Green Cross Corp. is currently producing an antihemophilic factor IX complex (FacNine) using conventional ion-exchange chromatography. The use of antihemophilic factor IX complex has been associated with a variety of thrombotic complications such as venous thromboembolism, myocardial infarction, pulmonary embolism, and disseminated intravascular coagulation. The cause of the thrombogenicity of the factor IX complex has proven to be thrombogenic impurities [21, 25]. These impurities include activated forms of vitamin K-dependent clotting factors II, VII, and X, and coagulant active phospholipids. Therefore, Green Cross Corp. has developed a high-purity factor IX concentrate free from thrombogenic materials. The purification process involves cryoprecipitation, DEAE-Sephadex A-50 anion-exchange chromatography, DEAE-Toyopearl 650 M anion-exchange column chromatography, heparin-Sepharose 6FF affinity column chromatography, and CM-Sepharose FF cation-exchange column chromatography. In order to increase the viral safety of the high-purity factor IX, particularly in regard to HAV and B19, a virus removal process using a polyvinylidene fluoride membrane filter (Viresolve NFP) has been optimized. The Viresolve NFP filter is specifically designed to remove B19 and HAV from high-purity proteins [6, 18]. In this paper, the effect of operating process parameters such as pH, temperature, and pressure on the filtration and recovery of factor IX was evaluated. In addition, the efficiency of the filter for removing several experimental model viruses for human pathogenic viruses, including HAV, porcine parvovirus (PPV), murine encephalomyocarditis virus (EMCV), HIV, bovine viral diarrhea virus (BVDV), and bovine herpes virus (BHV), was studied.

Materials and Methods

Preparation of High-Purity Factor IX

High-purity factor IX concentrate has been developed by Green Cross Corp., under the name GreenNine VF. The high-purity factor IX solution used in this study was prepared by cryoprecipitation, DEAE-Sephadex A-50 anion-exchange chromatography, solvent/detergent treatment, DEAE-Toyopearl 650 M anion-exchange chromatography, heparin-Sepharose 6FF affinity chromatography, and CM-Sepharose cation-exchange chromatography (Fig. 1). The highly purified factor IX solution was obtained from the large-scale production batches, and stored below −70°C until use. The potency of the solution was about 168 IU of factor IX per mg protein. One IU of factor IX is the amount contained in 1 ml of normal human plasma.

Filtration Apparatus and Filtration Procedure

The optimal condition for factor IX filtration using Viresolve NFP filter was studied using a 47-mm disk membrane filter (effective filtration area: 13.8 cm^2). The Viresolve NFP disk membrane was soaked into Water for Injection (WFI) with the shine side faced up until the membrane layer became uniformly wet. A stainless steel 47-mm diameter disk filter holder (Millipore) with a 200-ml resovoir was used to house the Viresolve NFP disk filter (Fig. 2). The assembled membrane holder was submerged with WFI for 1 min at 2.7 bar and leak-checked. After the leak test, the membrane was pressure-flushed with 100 ml of WFI at 2 bar and conditioned with test solution buffer (50 ml of 20 mM sodium citrate buffer, pH 6) at a constant pressure of 2 bar. Then, the transmission of protein through the conditioned filter was tested. Approximately 25 ml of the solution was filtered through the membrane. The flow rate was checked as a function of time and recovery yield after filtration was measured.

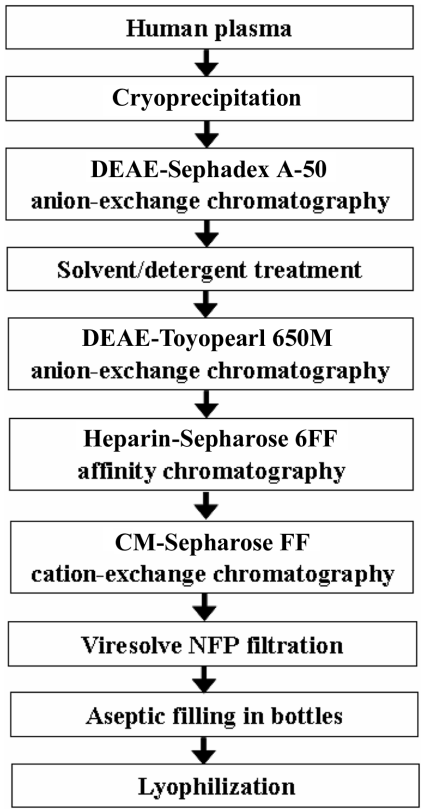

Fig. 1. Flow diagram of the manufacturing process for high-purity factor IX from human plasma.

Effect of Operating pH, Pressure, and Temperature on the Filtration Process

Factor IX solutions used for optimizing the virus filtration process was the eluate fraction from CM-Sepharose cation-exchange chromatography. The elution buffer used during CM-Sepharose cation-exchange chromatography was sodium citrate buffer (20 mM, pH 4.1) containing L-arginine (1 g/l) and NaCl (0.3 M). The pH of the solution was adjusted to 4, 5, 6, or 7 by the addition of HCl or NaOH. The optimum pH for the filtration of factor IX solution was measured using the pH-adjusted factor IX solutions. Factor IX solutions were prefiltered using 0.22-μm filters (Millex-W, Millipore) and then filtered through the Viresolve NFP filter under the constant pressure of 2 bar. Cumulative filtration volumes were measured as a function

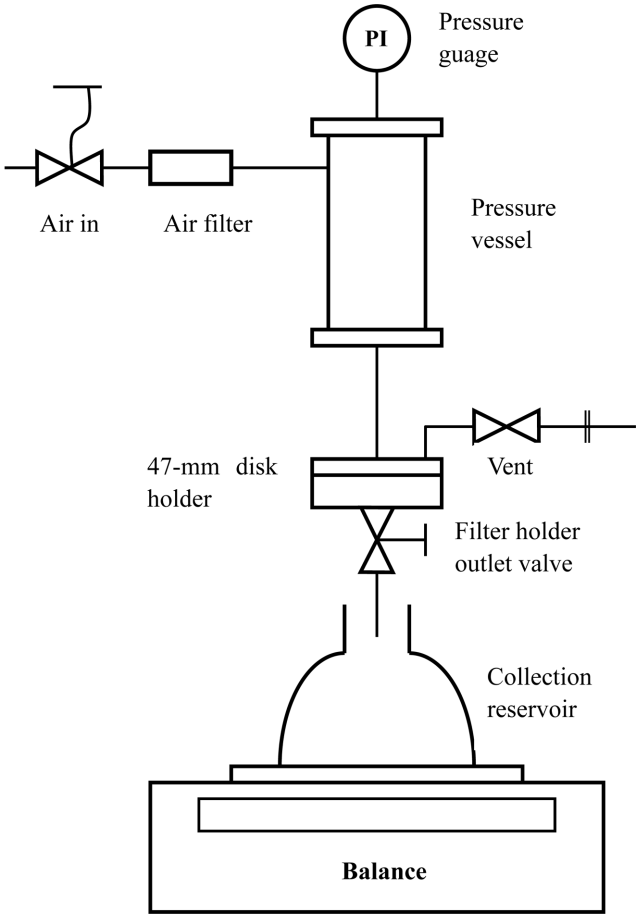

Fig. 2. Filtration apparatus for process optimization and virus validation study using a Viresolve NFP disk membrane.
The filtrate volume was recorded by measuring the filtrate mass using a balance.

of time. The effect of operating pressure on the filtration and recovery of factor IX solution at pH 6 was evaluated under the varying pressures of 1, 2, or 3 bar. The effect of operating temperatures (10, 15, and 20°C) on the filtration and recovery of factor IX solution at pH 6 was evaluated under the constant pressure of 2 bar. The temperature of the pressure vessel was controlled with a water jacket of each temperature.

Process Scale-Up

After process optimization using the 47-mm disk membrane filter, the Viresolve NFP cartridge filter (effective filter area: 4,800 cm^2) was used for scale-up to the manufacturing process. Several batches of this manufacturing process were analyzed to validate the process.

Validation of Scale-Down Process

The scale-downed process using a 47-mm disk membrane filter was operated under the same condition of the manufacturing process using the Viresolve NFP cartridge filter. Table 1 shows a typical comparison of some process parameters for the filtration processes. The validity of the scale-down was demonstrated by comparison of process parameters such as the concentration of protein and recovery yield of active ingredient.

Physical and Biochemical Analysis

All the physical and biochemical analyses were performed according to the Standard Operating Procedure (SOP) based on the Korean Pharmacopoeia, European Pharmacopoeia, and US Pharmacopoeia. The factor IX activity was determined using the clotting method with factor IX deficient plasma. Clotting times were determined on a KC10 coagulometer (Amelung, Lemgo, Germany). Abnormal toxicity was determined by the abdominal injection of a test sample into mice, with the subsequent monitoring of the toxicity and skin reactivity for up to 7 days post treatment. The presence of pyrogens was detected by administration of the test sample to the ear vein of rabbits, with subsequent monitoring for temperature changes [33].

Design of Worst-Case Condition for Virus Validation

It is necessary to show that all manufacturing viral clearance processes are effective under worst-case conditions. Therefore, it has been recommended that a virus validation study should be performed under worst-case conditions to demonstrate the minimum clearance a step can provide [2, 14]. As the worst-case condition for the filtration process, the highest throughput volume-to-surface area ratio was adopted (Table 1). Maximum working volume for the Viresolve NFP 20 cartridge during the production process has been set up to be 5,000 ml. From the specification, the maximum throughput volume for scale-down should be 14 ml. As the worst-case condition, a 5% surplus volume was added. Therefore, the working volume for the virus challenge experiment was 15.7 ml or more. The volume of the post-wash solution was another variable in the clearance evaluation studies. The worst-case would be the maximum wash volume, because that could maximize the filtration of viruses through the membrane. As the worst case condition, the working volume of the post-wash solution was 15.7 ml or more.

Validation of Removal of Viruses

The Viresolve NFP disk membrane was used to simulate the process performance of the production scale cartridges and to test if it could remove HAV, PPV, EMCV, HIV, BHV, and BVDV. The frozen

Table 1. Comparison of some typical process parameters for Viresolve NFP filtration during production runs, scale-down runs, and virus-challenge runs.

Item	Production runs	Scale-down runs	Virus-challenge runs
Effective surface area of virus filter (cm^2)	4,800	13.8	13.8
Working volume of test solution (ml)	5,000	14	15.7
Working volume of post-wash solution (ml)	5,000	14	15.7
Working temperature (°C)	20	20	20
Pressure for filtration (bar)	2.0	2.0	2.0

factor IX solution was thawed and warmed to 20°C. Factor IX solution was prefiltered using a 0.22-μm membrane (Millex-W, Millipore). This prefiltration was performed within 5 min prior to spiking viruses to the test solution. Twenty-seven ml of prefiltered factor IX solution was spiked with 3 ml of virus stock solution. After mixing, a sample was withdrawn, diluted with cell culture medium, and titrated immediately. The virus-spiked factor IX solution was prefiltered using a 0.22-μm membrane in order to remove viral aggregate, particulates, or viruses binding to proteins. A sample was withdrawn from the prefiltrate, diluted with cell culture medium, and titrated immediately. Subsequently, the prefiltrate was filtered through the disk membrane at a constant pressure of 2 bar to a maximum throughput volume of 15.7 ml of test solution. A sample was withdrawn from the filtrate, diluted with cell culture medium, and titrated immediately. After collecting 15.7 ml of test solution, the membrane was post-washed with 15.7 ml of the test solution buffer at a constant pressure of 2 bar. A sample was withdrawn from the post-wash solution, diluted with cell culture medium, and titrated immediately. After post-washing the virus filter, the post-integrity of the filter was tested by submerging the assembled membrane holder with water for 1 min at 2.7 bar and checking for leaks. Aseptic pressured air was used during the filtration.

Preparation and Titration of Viruses

In this study, HAV (strain HM/175/18f clone B, ATCC VR-1402), PPV (ATCC VR-742), and EMCV (ATCC VR-129B) were chosen as models of non-enveloped viruses, and HIV (IIIB; Advanced BiotechnoLogies, Columbia, MD, U.S.A.), BVDV (ATCC VR-534), and BHV (ATCC VR-188) were selected as examples of enveloped viruses. For the propagation and titration of HAV, PPV, EMCV, HIV, BVDV, and BHV, FRhK-4 (ATCC CRL-1688) cells, minipig kidney (MPK) cells (ATCC CCL-166), Vero C1008 cells (ATCC CRL-1586), C8166 cells (European Collection of Animal Cell Culture), bovine turbinate (BT) cells (ATCC CRL-1390), and Madin-Derby bovine kidney (MDBK) cells (ATCC CRL-22) were used, respectively, as described in previous reports [17–19, 35].

An aliquot from each sample from the virus inactivation studies and an appropriate control were titrated immediately after being collected in 7-fold serial dilutions to the end point using a quantal 50% tissue culture infectious dose ($TCID_{50}$) assay [15]. For the titration of HAV, EMCV, PPV, BHV, and BVDV, indicator cell monolayers in 24-well culture plates were 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. After 7–14 days of incubation, the wells were examined for cytopathic effect. For the titration of HIV, suspensions of C8166 cells in 96-well culture plates were infected using at least eight 0.1-ml replicates of the appropriate dilution of sample or positive control. Negative control wells were mock-infected using at least eight 0.1-ml replicates of culture medium. The plates were incubated at 35°C for approximately 1 h, and the wells were fed with 0.1 ml of the tissue culture medium. Approximately 14–21 days later, the wells were examined for any cytopathic effect and syncitial formation. As part of the virus validation protocol, cytotoxicity, interference, and load titer tests were performed. Cytotoxicity tests were performed on samples generated for virus titration in virus spiking experiments to control for possible cytotoxic effects on the indicator cells, which would interfere with the virus titration. Interference studies were performed to determine whether the test materials exert an inhibitory effect on the ability of the cell lines to permit detection of the virus. 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.

Calculation of Virus Reduction Factors

The virus reduction factor was defined as the $\log_{10}$ of the ratio of the virus loads in the spiked starting and post process materials, as described in a previous report [18]. All the virus inactivation experiments were carried out in duplicate and mean values are given.

RESULTS

Effect of Operating pH on the Filtration Process

As the first step to optimize the Viresolve NFP filtration process, the effect of operating pH on the filtration and recovery of factor IX was evaluated using a 47-mm disk membrane filter. Factor IX solutions of pH 4, 5, 6, or 7 were prefiltered using 0.22-μm filters and then filtered

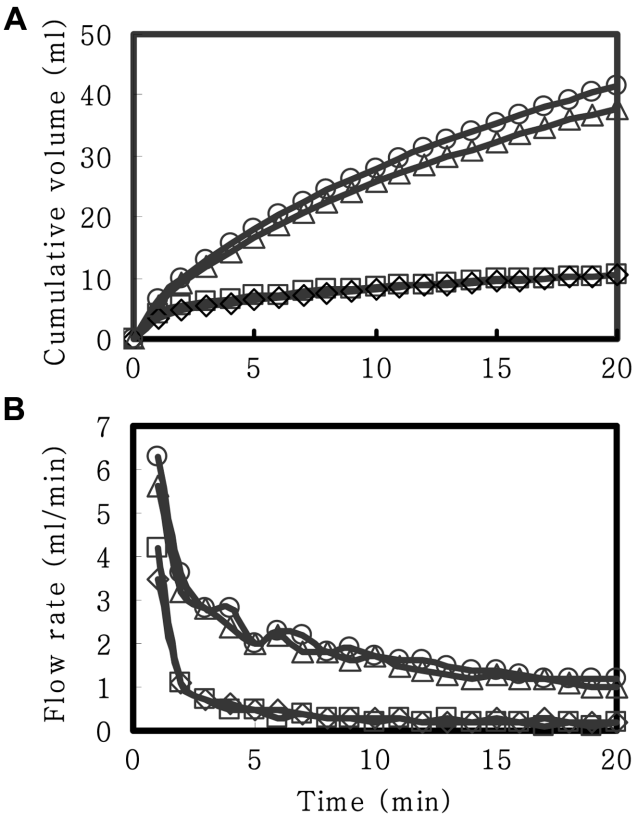

Fig. 3. Effect of operating pH on the cumulative filtration volume (**A**) and flow rate (**B**) of factor IX solution through Viresolve NFP disk membrane.

The pH of the process solution was 4 (◇), 5 (□), 6 (△), and 7 (○). Cumulative filtration volume and flow rate were measured as a function of time.

through the Viresolve NFP filter under the constant pressure of 2 bar. Cumulative filtration volumes were measured as a function of time (Fig. 3A). Flow rates at the lower pH values of 4 or 5 were much lower than those at pH values of 6 or 7 (Fig. 3B). Recovery yield was also pH-dependent. Recovery yields were 26.2% for pH 4, 27.7% for pH 5, 91.5% for pH 6, and 91.7% for pH 7. Flow rates and recovery yields were nearly the same at pH 6 and 7; however, the process time needed to increase the pH of factor IX solution from 4.1 to 7 was much longer. The longer process time and more addition of NaOH solution caused slight inactivation of factor IX. Hence, pH 6 was selected as the operating pH for the virus filtration process.

Effect of Operating Pressure on the Filtration Process

The effect of operating pressure on the filtration and recovery of factor IX was evaluated. Factor IX solutions at pH 6 were prefiltered using 0.22-μm filters and then filtered through the Viresolve NFP filter under the varying pressures of 1, 2, or 3 bar. Cumulative filtration volumes were measured as a function of time during filtration (Fig. 4A). Flow rates during filtration were dependent on operating pressure. As the operating pressure increased, the flow rate increased (Fig. 4B). However, the recovery yields were not changed by varying the operating pressure under these experimental conditions. Recovery yields were 91.6% for pressure of 1 bar, 91.5% for pressure of 2 bar, and 91.7% for pressure of 3 bar. Maintaining the pressure constantly at 2 bar was easier than at 3 bar. Therefore, 2 bar was chosen as the operating pressure for the virus filtration process.

Effect of Operating Temperature on the Filtration Process

The effect of the operating temperature on the filtration and recovery of factor IX was evaluated. Factor IX solutions at pH 6 were warmed up to 10, 15, or 20°C. The solutions were prefiltered using 0.22-μm filters and then filtered through the Viresolve NFP filter under the constant pressure of 2 bar. Cumulative filtration volumes were measured as a function of time during filtration (Fig. 5A). Flow rates during filtration were dependent on the operating temperature. As the operating temperature increased, the flow rate increased (Fig. 5B).

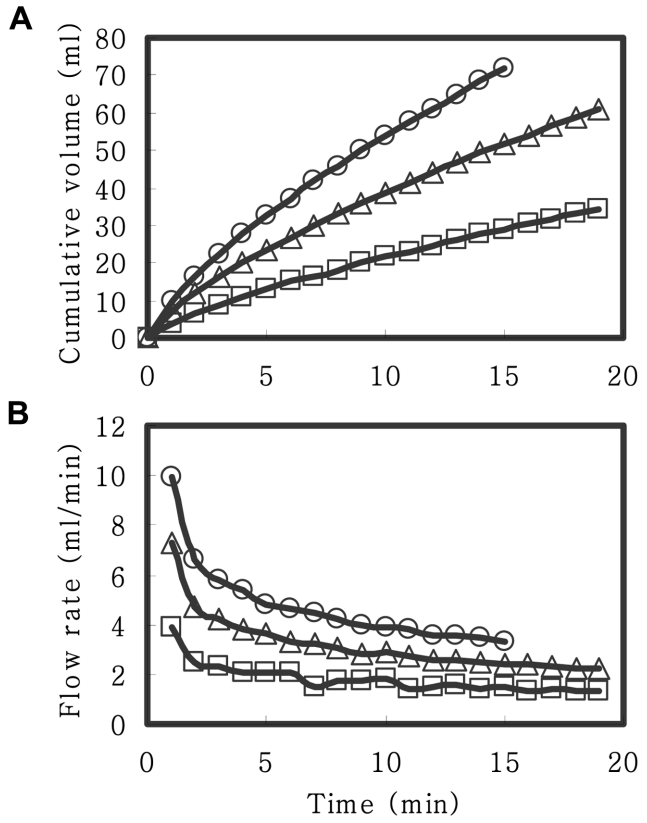

Fig. 4. Effect of operating pressure on the cumulative filtration volume (**A**) and flow rate (**B**) of factor IX solution through Viresolve NFP disk membrane.

The operating pressure was 1 bar (□), 2 bar (△), and 3 bar (○). Cumulative filtration volume and flow rate were measured as a function of time.

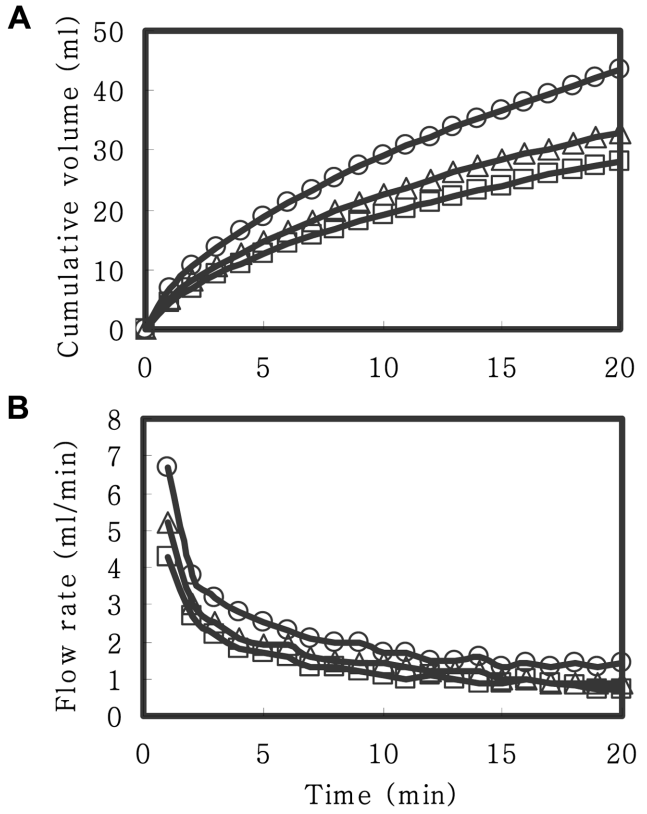

Fig. 5. Effect of operating temperature on the cumulative filtration volume (**A**) and flow rate (**B**) of factor IX solution through Viresolve NFP disk membrane.

The operating temperature was 10°C (□), 15°C (△), and 20°C (○). Cumulative filtration volume and flow rate were measured as a function of time.

Table 2. Recovery of factor IX activity and protein content after filtration using Viresolve NFP cartridge

Production batch No.	Total factor IX activity (IU)		Total protein content (mg)	
	before	after	before	after
1	613,500	585,208 (94.9)[a]	3,805	3,584 (94.2)
2	668,500	643,529 (96.3)	3,690	3,513 (95.2)
3	621,615	599,237 (96.4)	3,813	3,657 (95.9)

[a]These values in parentheses indicate % recovery yields after filtration.

Recovery yields also increased as the operating temperature increased. Recovery yields were 83.7% at 10°C, 86.5% at 15°C, and 92.1% at 20°C. Therefore, 20°C was selected as the operating temperature for the virus filtration process.

Process Scale-Up and Process Validation

Viresolve NFP filtration was scaled-up to the manufacturing scale using a cartridge filter of which the effective surface area is 4,800 cm^2. Five thousand ml of a process solution was prefiltered using a 0.22-μm prefilter and then filtered through the Viresolve NFP cartridge filter at a constant pressure of 2 bar and 20°C. The recovery yield was about 92% after filtration. After filtration, 5,000 ml of post-wash buffer was filtered in order to wash out remaining factor IX protein in the cartridge filter. The increased recovery yield after additional post-washing procedures was about 4%. Therefore, the total recovery yield was 96%.

In order to assure that the Viresolve NFP filtration process will consistently operate and produce a product of the required quality, a process validation study was conducted (Table 2). The analysis of three batch operations showed that this filtration process has consistency and reproducibility. There was no batch-to-batch variation in terms of recovery yields of factor IX activity and protein.

Effect of Filtration upon Factor IX Characteristics

The influence of Viresolve NFP filtration upon factor IX characteristics was studied (Table 3). There was no change in appearance of the solution. Specific activity was not changed during filtration. Activation of factor IX was not detected before and after filtration. Abnormal toxicity against mouse was not induced after filtration. Furthermore, the concentration of pyrogenic substances was not changed.

Table 3. Characteristics of factor IX before and after filtration.

Parameters	Before filtration	After filtration
Specific activity (IU/mg)	168.5±11.0	170.1±11.3
Activated factor IX	Not detected	Not detected
Abnormal toxicity against mice	Not detected	Not detected
Pyrogen (°C)	0.55±0.05	0.51±0.06

Removal of Non-Enveloped Viruses (HAV, PPV, and EMCV) During Filtration

To evaluate the effectiveness and robustness of the Viresolve NFP filtration process in eliminating small, non-enveloped viruses, two different lots of Viresolve NFP disk membranes were challenged with HAV, PPV, and EMCV (Table 4). No infectious viruses were detected in the filtrate of any of the two filter lots tested, indicating that these viruses were completely removed to below the detection level. The average log reduction factors achieved were ≥6.12 for HAV, ≥4.28 for PPV, and ≥5.33 for EMCV.

Removal of Enveloped Viruses (HIV, BVDV, and BHV) During Filtration

To determine whether large, enveloped viruses could be eliminated during filtration, two different lots of Viresolve NFP disk membranes were challenged with HIV, BVDV and BHV (Table 5). No infectious viruses were detected in the filtrate of any of the two filter lots tested, indicating that these viruses were completely removed to below the detection level. The average log reduction factors achieved were ≥5.51 for HIV, ≥5.17 for BVDV, and ≥5.75 for BHV.

DISCUSSION

Although antihemophilic factor IX has been regarded as generally safe against viruses, which is attributed to

Table 4. Removal of PPV, HAV, and EMCV during Viresolve NFP filtration.[a]

Sample	Total virus titer (log_{10} $TCID_{50}$)		
	HAV	PPV	EMCV
Factor IX solution spiked with virus	7.50	5.66	6.71
Filtrate after Viresolve NFP filtration	ND[b] (≤1.38)[c]	ND (≤1.38)	ND (≤1.38)
Post-washed solution	ND (≤1.38)	ND (≤1.38)	ND (≤1.38)
Reduction factor (log_{10})	≥6.12	≥4.28	≥5.33

[a]These results are mean values of two independent experiments.
[b]No infectious viruses were detected.
[c]These values were calculated using a theoretical minimum detectable level of infectious virus with a 95% confidence level.

Table 5. Removal of HIV, BVDV, and BHV during Viresolve NFP filtration.[a]

Sample	Total virus titer (log_{10} $TCID_{50}$)		
	HIV	BVDV	BHV
Factor IX solution spiked with virus	6.41	6.55	7.13
Filtrate after Viresolve NFP filtration	ND[b] (≤0.90)[c]	ND (≤1.38)	ND (≤1.38)
Post-washed solution	ND (≤0.90)	ND (≤1.38)	ND (≤1.38)
Reduction factor (log_{10})	≥5.51	≥5.17	≥5.75

[a]These results are mean values of two independent experiments.
[b]No infectious viruses were detected.
[c]These values were calculated using a theoretical minimum detectable level of infectious virus with a 95% confidence level.

the natural clearance effect during the purification process and to the viral inactivation step such as the solvent/detergent treatment, a concern regarding the virus safety of this product against non-enveloped viruses such as HAV and parvovirus has been raised because of these viruses' high resistance to physicochemical treatment [9, 12, 27, 30]. For this reason, implementation of multiple viral clearance (inactivation and/or removal) steps has been highly recommended for manufacturing of plasma-derived biopharmaceuticals. Of the available viral clearance strategies, virus-retentive filtration is often a method of choice, being considered a robust technique not highly susceptible to minor changes in process conditions [2, 8].

Viresolve NFP membrane is a high-flux composite membrane that uses a size-exclusion mechanism [6, 18]. The positioning of a virus removal step is governed by several considerations including product characteristics (protein concentration, degree of purity, *etc.*) that will influence flux rates and products transmission, the nature of the viral contaminants, and the necessity to document the exclusion of adventitious viruses that may be introduced during production [3, 8]. Generally, the protein concentration of the feed solution and the presence of impurities in the feed solution can affect the virus filtration process by reducing product throughput. For optimizing virus filter performance, the virus filtration process was positioned after the CM-Sepharose cation-exchange chromatography process, which is the final chromatographic polishing step for purifying the high-purity factor IX from human plasma. The purity of factor IX after CM-Sepharose cation-exchange chromatography was 11,300-fold higher than that of human plasma (data not shown). This high purity of feed solution can reduce the process time and increase product yield. Usually, a high concentration of feed protein causes the membrane to become polarized and a drop in product throughput [8].

Virus filter capacity is affected by plugging aggregates in the feedstock. The nature and quantity of aggregates depends on the protein, its purity and source, buffer conditions, hold time, and temperature. Therefore, prefiltration of the feed solution can have a dramatic impact on filter performance [2, 6, 18]. Prefiltration is targeted to remove various impurities or contaminants, such as protein aggregates, DNA, and other trace materials. In order to remove impurities or contaminants, and then increase the capacity of the virus filter, factor IX solution was prefiltered using an 0.22-μm membrane (Millex-W, Millipore) before Viresolve NFP filtration.

The most critical factor affecting the filtration efficiency was operating pH. Flow rates and recovery yields at the lower pH values of 4 or 5 were much lower than those at pH 6 or 7, which was probably due to aggregation of the proteins. At these lower pH conditions, factor IX was aggregated, because the isoelectric points of factor IX were between 4.1 and 4.6, depending on the patterns of glycosylation [36]. Aggregated factor IX will be more effectively retained on the filter.

The loss of yield during filtration can occur through hold-up of fluid in the filtration device or supporting equipment. Fluid hold-up in the filtration equipment is usually handled by flushing the system with buffer to recover the protein, and by sizing and designing the system so that hold-up is small enough to be acceptable. The recovery yield of factor IX was about 92% after filtration. Further post-washing of the filtration system with buffer increased the product yield up to 96%. Another factor causing yield loss is protein degradation during filtration. The longer process time or higher operating temperature can induce protein degradation. SDS-PAGE of the factor IX before and after Viresolve NFP filtration did not reveal the emergence of new bands (data not shown), indicating that the process had not induced cleavage. The operating time required for one batch solution of 5,000 ml to be filtered through the Viresolve NFP cartridge was only 20 min. A high process flow rate can provide several benefits by reducing process time, installed membrane area, product loss, and production space.

Both the specific activity and the fraction of activated factor IX were not changed after filtration, indicating that Viresolve NFP filtration did not alter the biological activity of the protein. In addition, abnormal toxicity against mice was not induced during filtration and the concentration of pyrogenic substances was not changed after filtration. These results showed that the filtration is a fairly simple process, inducing no detectable effects on the physical and biochemical characteristics of factor IX.

A validation study was designed to evaluate the efficacy of the Viresolve NFP filter for removal of viruses. A range of virus sizes and non-enveloped or enveloped viruses were employed in order to fully evaluate the filter's performance. HAV is a member of the Picornaviridae family, which is a 25–30 nm, non-enveloped, and single-stranded RNA virus with a medium to high resistance to physicochemical inactivation. PPV was chosen as a model virus for B19, which is one of the smallest viruses found in human serum. PPV is an 18–26 nm, non-enveloped, and single-stranded DNA virus, which exhibits a high resistance to physicochemical inactivation. EMCV is a 28–30 nm, non-enveloped, and single-stranded RNA virus with a medium to high resistance to physicochemical inactivation. BVDV is a 40–60 nm, enveloped, and single-stranded RNA virus with a medium resistance to physicochemical inactivation. BVDV belongs to the Flaviviridae family, which also includes the hepatitis C and hepatitis G viruses. BHV is a 120–300 nm, enveloped, and double-stranded DNA virus. BHV is a surrogate for the human herpesvirus, such as HHV-6, HHV-7, HHV-8, Epstein Barr virus, or HSV-1 [1, 24].

The quality of the virus stocks, as measured by the presence of viral aggregates, cell debris, or other particulates, can influence results by falsely enhancing or reducing viral clearance [2]. For example, in direct flow filtration, a membrane prematurely clogged by cell debris cannot filter the entire load volume. Viral aggregation, whether due to the virus stock preparation, viruses attaching membrane particulates or host cells, or viruses binding to proteins in the test solution, enhances the apparent retentive capacity of a filter and provides false clearance values. Therefore, prefilters should be used before virus removal filtration to remove virus aggregates or debris that can falsely increase clearance. Prefiltration can use sterilizing-grade (0.1 or 0.22 μm) membrane. For this study, a 0.22-μm membrane was used as a prefilter. The results presented in the present study showed that all the viruses tested were completely removed during the Viresolve NFP filtration process. These results demonstrate that the Viresolve NFP filter is effective for removing smaller size viruses of HAV, PPV, and EMCV as well as HIV, BVDV, and BHV from factor IX solution. Considering that parvovirus is one of the smallest viruses found in human fluid, this filter shows potential for increasing the safety of biological products where non-enveloped viruses, including parvovirus and HAV, are of concern.

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