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Optimization and Validation of a Virus Filtration Process for Efficient Removal of Viruses from Urokinase Solution Prepared from Human Urine

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Abstract Urokinase is an enzyme with fibrinolytic activity (plasminogen activator) isolated from fresh urine of healthy men. Viral safety is an important prerequisite for clinical preparation of the protein from urine. In order to increase the viral safety of a high purity urokinase in regard to non-enveloped viruses, a virus removal process using a novel polyvinylidene fluoride membrane filter (Viresolve NFP) has been optimized. Urokinase was able to pass through the filter with recoveries of 95% in the production scale process. No substantial changes were observed in physical and biochemical characteristics of the filtered urokinase in comparison with those of the enzyme before filtration. A 47-mm disk membrane filter was used to simulate the process performance of the production scale cartridges and tested if it could remove several experimental model viruses for human pathogenic viruses, including porcine parvovirus (PPV), human hepatitis A virus (HAV), murine encephalomyocarditis virus (EMCV), bovine viral diarrhoea virus (BVDV), and bovine herpes virus (BHV). Non-enveloped viruses (PPV, HAV, and EMCV) as well as enveloped viruses (BVDV and BHV) were completely removed during filtration. The log reduction factors achieved were ≥4.86 for PPV, ≥4.60 for HAV, ≥6.87 for EMCV, ≥4.60 for BVDV, and ≥5.44 for BHV. These results indicate that the virus filtration process successfully improved the viral safety of the final products.

Key words: Urokinase, parvovirus, hepatitis A virus, virus removal, virus filtration, validation

Urokinase is an enzyme with fibrinolytic activity (plasminogen activator) isolated from fresh urine of healthy men. It consists of a mixture of low-molecular mass (33 kDa) and high-molecular mass (54 kDa) forms, the latter form being predominant. Urokinase has been reported to be effective for cerebral thrombosis, myocardial infarction, and arteriovenous thrombosis in limbs, and to potentiate the effect of anti-tumor drugs [8, 26, 28]. Although urokinase has been highly purified for clinical application, special precautions must be taken during the production of this protein to assure against the possibility of the product transmitting infectious diseases to the recipients, because urokinase is manufactured from human urine. The possible risks associated with the use of urokinase are viral infections such as hepatitis A virus (HAV), cytomegalovirus, and other bloodborne viruses [6, 9, 18, 25].

Viral safety of biopharmaceuticals cannot be ensured only by demonstration of absence of infectious contaminants in a product. One approach to prevent virus transmission is to screen the starting material for virus. However, such an approach requires a specific test for each virus. Thus, additional procedures for the inactivation and removal of viruses, preferably effective against a wide range of virus types, must also be incorporated into the manufacturing process [2, 14]. Although many of these have been highly effective in controlling enveloped viruses such as human immunodeficiency virus, and hepatitis B and C, they are often less effective against non-enveloped viruses. For instance, parvovirus and HAV are resistant to pasteurization and solvent/detergent treatment [12–16, 23].

One potential way to increase the safety of therapeutic biological products is the use of virus-retentive filters. There have been several reports about the application of virus filters for removing possible contaminating viruses. However, most of these reports were focused on the removal of viruses ranging from 120 to 35 nm in size, because of the technical problems of making smaller-size filters [3, 4, 10, 11, 20, 21, 24, 27]. Recently, Millipore's Viresolve NFP filter of pore-size 20 nm was marketed, which is specifically designed to remove parvovirus and HAV from high purity proteins. From the developmental

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stage of the filter, the ability of this filter to remove viruses has been evaluated. In the present study, a virus removal process using the novel polyvinylidene fluoride membrane filter was optimized in order to increase the viral safety of a high purity urokinase in regard to non-enveloped viruses as well as enveloped viruses. In addition, the efficiency of the filter for removing several experimental model viruses for human pathogenic viruses, including porcine parvovirus (PPV), human hepatitis A virus (HAV), murine encephalomyocarditis virus (EMCV), bovine viral diarrhoea virus (BVDV), and bovine herpes virus (BHV), was evaluated.

MATERIALS AND METHODS

Urokinase Manufacturing Process and Positioning of Virus Filtration Process

Urokinase for clinical use was highly purified using PAB (para-amino benzamidine) affinity column chromatography from a bulk material prepared by a successive adsorption and extraction process using bentonite and calcium phosphate. After PAB chromatography, the purified urokinase was desalted using Sephadex G-25 column chromatography. The desalted urokinase solution was stabilized with 0.4% (w/v) albumin and pasteurized at 60±0.5°C for 10 h to inactivate any contaminating viruses. The pasteurized urokinase solution was put in bottles and lyophilized for clinical use. As the first step to choose an appropriate position of the Viresolve NFP filtration in the purification process for optimal product recovery as well as virus removal, the feasibility of the filter was studied using two process solutions. Process Solution I was the desalted urokinase solution whose protein concentration was 2.3 mg/ml. Process Solution II was the urokinase solution containing albumin as the stabilizer for pasteurization; its protein concentration was 4.4 mg/ml (Fig. 1).

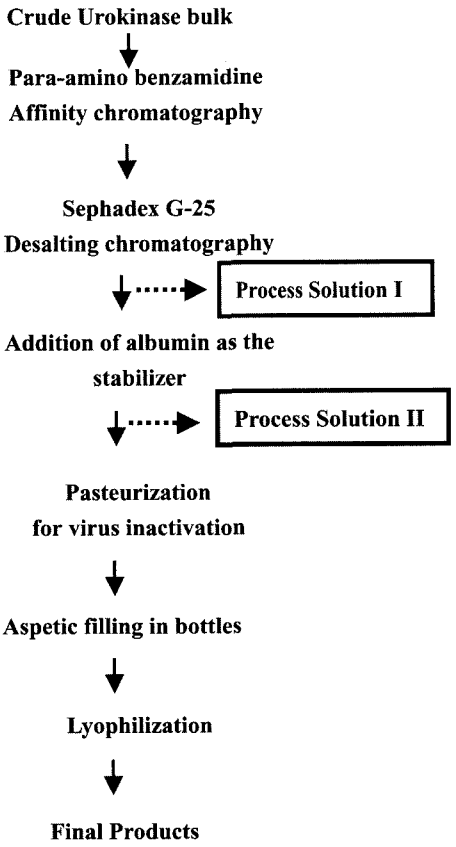

Fig. 1. Flow diagram of the manufacturing process for urokinase. The boxes indicate the process solutions evaluated for feasibility test and positioning of Viresolve NFP filter for the urokinase process. Process Solution I was the desalted urokinase solution with a protein concentration of 2.3 mg/ml. Process Solution II was the urokinase solution containing albumin as the stabilizer for pasteurization; its protein concentration was 4.4 mg/ml.

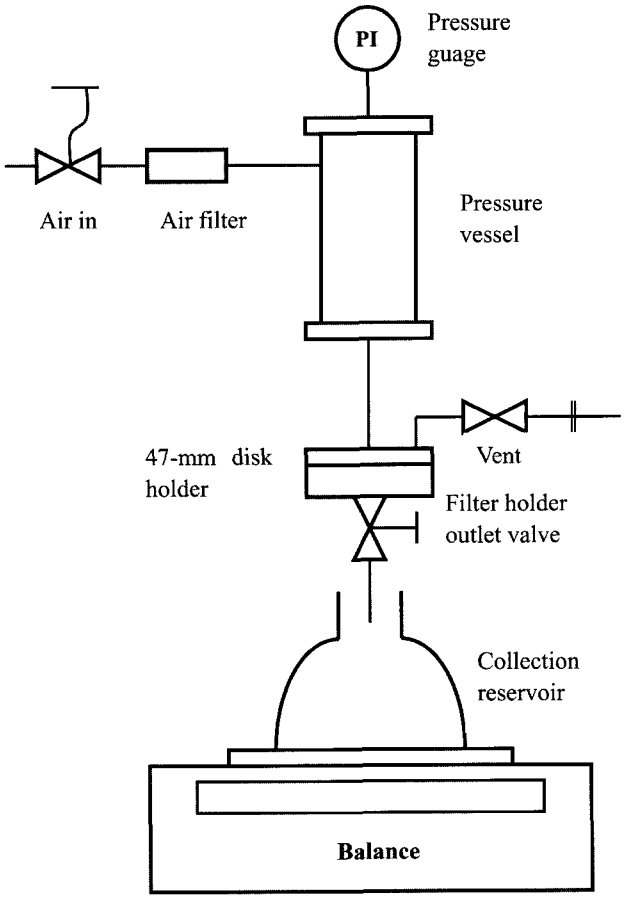

Fig. 2. Filtration apparatus for feasibility test and virus validation study using a Viresolve NFP disk membrane.
The filtrate volume was recorded by measuring filtrate mass using a balance.

Filtration Apparatus and Filtration Procedure

The feasibility of the 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

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)	3,500±500	10.1±1.4	12.1
Working volume of post-wash solution (ml)	350	1	1.2
Working temperature (°C)	2-8	2-8	2-8
Pressure for filtration (bar)	2.0	2.0	2.0

soaked with Water for Injection (WFI) with the shiny side face up until the membrane layer became uniformly wet. A stainless steel 47-mm diameter disk filter holder (Millipore) with a 200-ml reservoir was used to house the Viresolve NFP disk filter (Fig. 2). The assembled membrane holder was submerged in WFI for 1 min at 2.7 bar and leak was 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, prepared from sodium dihydrogen phosphate dihydrate (10.4 g/l) and disodium hydrogen phosphate dodecahydrate (1.04 g/l) dissolved in WFI] 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.

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

Determination of V_{max}

V_{max} represents the maximum volume of fluid that will pass through a filter before it is completely plugged. V_{max} is the preferred filter sizing method for constant pressure and normal flow filtration process [5]. In a V_{max} test, the challenge solution was filtered through the test device, and the cumulative volume was recorded as a function of time at a selected differential pressure. Time, T, in minutes was divided by cumulative volume, V, in milliliters, and time/volume was plotted versus time. For determining V_{max}, the data collected over a 10-30 min period were used. If a linear plot of time/volume versus time was obtained, it was assumed that the solution followed the gradual pore-plugging model. Slope, y-intercept, and correlation efficient were obtained by a least-squares-fit analysis of the linear plot. V_{max} was calculated as the inverse slope of this graph. The initial flow rate (ml/min) was calculated as the reciprocal of the y-intercept.

Validation of Scale-Down Process

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

Physical and Biochemical Analyses

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 potency of urokinase was determined by comparing its capacity to activate plasminogen to form plasmin with the same capacity of a reference preparation of urokinase calibrated in International Units. Abnormal toxicity was determined by the abdominal injection of a test sample in mice, with the subsequent monitoring of toxicity and skin reactivity for up to seven days post-treatment. The molecular fraction was calculated as the % ratio of the urokinase activity in the high-molecular mass fraction to that in the total urokinase fraction (high-molecular mass fraction plus low-molecular mass fraction) after size-exclusion chromatography. The presence of pyrogens was detected by administration of the test sample to the ear vein of rabbits, with subsequent monitoring for changes in temperature.

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]. As the worst-case condition for 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 was setup to be 4,000 ml. From the specification, the maximum throughput volume for scale-down should be 11.5 ml. As the worst-case condition, 5% surplus volume was added. Therefore, the working volume for virus challenge experiment was 12.1 ml or more. The volume of post-wash solution was another variable in 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 post-wash solution was 1.2 ml or more.

Validation of Removal of Viruses

Viresolve NFP disk membrane was used to simulate the process performance of the production scale cartridges and tested if it could remove PPV, HAV, EMCV, BVDV, and BHV. The frozen urokinase solution was thawed and warmed to 2 to 8°C. Urokinase solution was pre-filtered using 0.1 μm membrane (Millex-W, Millipore). This pre-filtration was performed within 5 min prior to spiking viruses to the test solution. Eighteen ml of pre-filtered urokinase solution was spiked with 2 ml virus stock solution. After mixing, a sample was withdrawn, diluted with cell culture medium, and titrated immediately. The virus-spiked urokinase solution was pre-filtered using 0.22 μm Millex-GV membrane (Millipore) in order to remove viral aggregate, particulates, host cells, or viruses binding to proteins. A sample was withdrawn from the pre-filtrate, diluted with cell culture medium, and titrated immediately. Subsequently, the pre-filtrate was filtered through the disk membrane at a constant pressure of 2.0 bar to a maximum throughput volume of 12.1 ml of test solution. A sample was withdrawn from the filtrate, diluted with cell culture medium and titrated immediately. After collecting 12.1 ml of the test solution, the membrane was post-washed with 1.2 ml of the test solution buffer at a constant pressure of 2.0 bar. A sample was withdrawn from the post-wash solution, diluted with cell culture medium and titrated immediately. After post-washing the virus filter, post-integrity of the filter was tested by submerging the assembled membrane holder in water for 1 min at 2.7 bar and checking for leaks. Aseptic pressured air was used during filtration and the virus filtration process was done in a cold chamber at 2 to 8°C.

Preparation and Titration of Viruses

In this study, PPV (ATCC VR-742), HAV (strain HM/175/18f clone B, ATCC VR-1402), and EMCV (ATCC VR-129B) were chosen as models of non-enveloped viruses, whereas BVDV (ATCC VR-534) and BHV (ATCC VR-188) were selected as examples of enveloped viruses. For the propagation and titration of PPV, HAV, EMCV, BVDV, and BHV, minipig kidney (MPK) cells (ATCC CCL-166), FRhK-4 (ATCC CRL-1688) cells, Vero C1008 cells (ATCC CRL-1586), 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 [15, 16]. Virus removal by the filter was expressed as a log reduction factor calculated by subtracting the log titer obtained after filtration from that obtained prior to filtration.

RESULTS

Feasibility Test and Positioning of Viresolve NFP Filter for Urokinase Process

As the first step to choose an appropriate position of the Viresolve NFP filtration in the purification process for optimal product recovery as well as viral removal, the feasibility of the filter was studied using a 47-mm disk membrane filter with an effective surface area of 13.8 cm^2. Test solutions were pre-filtered using a 0.22 μm filter and then filtered through the Viresolve NFP filter under the constant pressure of 2 bar. The cumulative filtration volume

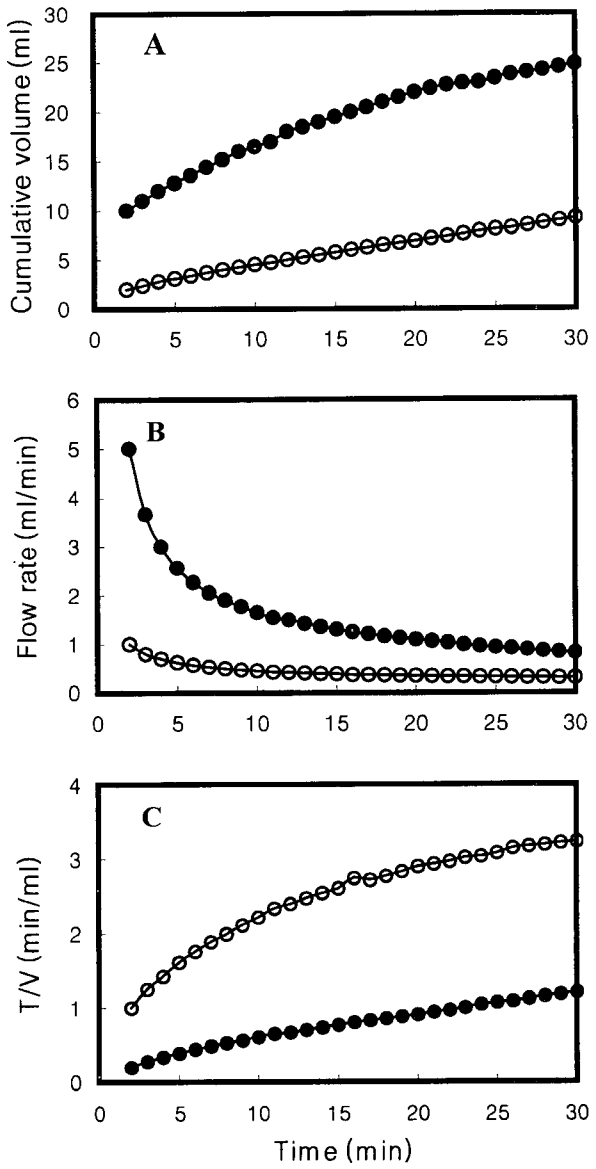

Fig. 3. Filterability of Process Solution I (●) and Process Solution II (○) through Viresolve NFP disk membrane.
(A) Cumulative filtration volume, (B) flow rate, and (C) time/volume were measured as a function of time.

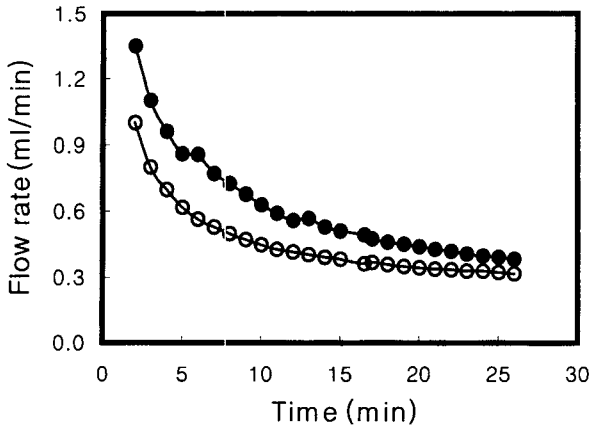

Fig. 4. Effect of pre-filtration condition on the flow rate of Process Solution II through Viresolve NFP disk membrane.
Process Solution II was pre-filtered using 0.1 μm pre-filter (●) and 0.22 μm pre-filter (○), respectively.

was measured as a function of time during filtration (Fig. 3A). Flow rates during filtration were dependent on protein concentrations of test samples. The flow rate of Process Solution I was much higher than that of Process Solution II (Fig. 3B). Time, T, in minutes, was divided by cumulative volume, V, in milliliters, and time/volume was plotted versus time (Fig. 3C). From the plot analysis, V_{max} per 47-mm disk membrane were predicted to be 33.22 ml for Process Solution I and 20.24 ml for Process Solution II. The initial flow rates calculated were 7.89 ml/min for Process Solution I and 1.24 ml/min for Process Solution II.

Also, the recovery yield of Process Solution I was much higher than that of Process Solution II. The recovery yields were 89% for Process Solution I and 84% for Process Solution II in this experimental condition.

The effect of pre-filtration on the flow rate and recovery yield was compared using 0.1 μm pre-filter and 0.22 μm pre-filter. Process Solution II was pre-filtered using a 0.1 μm pre-filter and 0.22 μm pre-filter, respectively, and then filtered through the Viresolve NFP filter under the constant pressure of 2 bar. As shown in Fig. 4, the flow rate of the test solution after 0.1 μm pre-filtration was much higher than that after 0.22 μm pre-filtration. Also, the recovery yield after 0.1 μm pre-filtration was more than 3% higher than that after 0.22 μm pre-filtration. The recovery yields were 87% for 0.1 μm pre-filtration run and 84% for 0.22 μm pre-filtration run.

The calculated results showed that flux and capacity of the virus filter could be limited by protein concentration and pre-filtration condition of the challenged solution. From the above results, Process Solution I was chosen as the target for the virus filtration process and the 0.1 μm pre-filtration process was adopted for higher recovery yield.

Process Scale-Up and Process Validation

Viresolve NFP filtration was scaled-up to the manufacturing scale using a cartridge filter with an effective surface area of 4,800 cm^2. Three-thousand-and-five-hundred ml of a Process Solution I was pre-filtered using a 0.1 μm pre-filter and then filtered through the Viresolve NFP cartridge filter at a constant pressure of 2 bar. The recovery yield was about 92% after filtration. After filtration, 350 ml of post-wash buffer was filtered in order to wash out the remaining urokinase protein in the cartridge filter. The increased recovery yield after the additional post-washing procedure was about 3%. Therefore, the total recovery yield was 95%.

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 batches of operation showed that this filtration process had consistency and reproducibility. There was no batch-to-batch variation in terms of recovery yields of urokinase activity and protein.

Effect of Filtration upon Urokinase Characteristics

The influence of Viresolve NFP filtration upon urokinase characteristics was studied (Table 3). There was no change in appearance of the solution. Specific activity was not changed during filtration. The ratio of the urokinase activity in the high-molecular-mass fraction to that in the total urokinase fraction was not changed. Abnormal toxicity against mice was not induced after filtration. Also, the concentration of pyrogenic substances was not changed.

The next step after Viresolve NFP filtration was a pasteurization process. In order to learn the possible negative effect of the virus filtration process on pasteurization, the recovery yield of urokinase activity after pasteurization of the filtered solution was compared with that of urokinase solution without filtration. The average recovery yield of

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

Production batch No.	Total urokinase activity (IU)		Total protein content (mg)	
	Before	After	Before	After
1	1,484,340,000	1,421,040,000 (95.7)[a]	7,854	7,525 (95.8)
2	1,407,250,000	1,332,090,000 (94.7)	8,255	7,870 (95.3)
3	1,461,600,000	1,395,000,000 (95.4)	7,627	7,291 (95.5)

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

Table 3. Characteristics of urokinase before and after filtration.

Parameters	Before filtration	After filtration
Specific activity (IU/mg)	183,695±11,528	183,142±12,081
Molecular fraction (%)	99.77±0.06	99.30±0.06
Abnormal toxicity against mouse	Negative	Negative
Pyrogen (°C)	0.55±0.05	0.51±0.06

urokinase solution with filtration was 93%, which was the same as that of urokinase solution without filtration. This result indicates that the virus filtration process does not affect the pasteurization step.

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

To evaluate the effectiveness and robustness of the Viresolve NFP filtration process in eliminating small, non-enveloped viruses, three different lots of Viresolve NFP disk membranes were challenged with PPV, HAV, and EMCV (Table 4). No infectious viruses were detected in the filtrate of any of the three filter lots tested, indicating that these viruses were completely removed to below detection level. The average log reduction factors achieved were ≥4.86 for PPV, ≥4.60 for HAV, and ≥6.87 for EMCV.

Removal of Enveloped Viruses (BVDV and BHV) During Filtration

To determine whether large, enveloped viruses could be eliminated during filtration, three different lots of Viresolve NFP disk membranes were challenged with BVDV and BHV (Table 5). No infectious viruses were detected in the filtrate of any of the three filter lots tested, indicating that these viruses were completely removed to below detection level. The average log reduction factors achieved were ≥4.60 for BVDV and ≥5.44 for BHV.

DISCUSSION

Although urokinase has generally been regarded as safe against viruses, which is attributed to the natural clearance effect during the purification process and to the viral inactivation step such as pasteurization, a concern regarding the virus safety of this product against non-enveloped viruses such as parvovirus and HAV has been raised, because of these viruses' high resistance to physicochemical treatment [7, 12, 14, 19, 22]. For this reason, implementation of multiple viral clearance (inactivation and/or removal) steps has been highly recommended for manufacturing urine-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. Filtration for virus removal has several advantages over other conventional process steps that may demonstrate viral clearance capability [2]. Removal by size exclusion is not directly influenced by filtration conditions (e.g., differential pressure, temperature, viral challenge level, etc.) and characteristics of the products to be filtered (e.g., viscosity, ionic strength, pH, surface tension, etc.). Additionally, filtration is one of the least invasive processes; it does not require inclusion of stabilizers or other additives that have a potential for toxicity; it does not alter the antigenicity of the target protein or induce the formation of neoantigens, and, in general, it is very amenable to inclusion into the manufacturing process.

The positioning of a virus removal step is governed by several considerations including product characteristics (protein concentration, degree of purity, etc.) which can influence flux rates and products transmission, the nature of the viral contaminants, and necessity to document exclusion of adventitious viruses that may be introduced during production [3]. The filterability data (flow rate and products transmission) indicate a higher flow rate for

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

Sample	Total virus titer (log_{10} $TCID_{50}$)		
	PPV	HAV	EMCV
Urokinase solution spiked with virus	6.63±0.44	6.62±0.34	7.05±0.32
Pre-filtered urokinase solution	6.42±0.46	6.62±0.40	6.62±0.44
Filtrate after Viresolve NFP filtration	ND[b] (≤1.77[c])	ND[b] (≤2.02[c])	ND[b] (≤0.18[c])
Post-washed solution	ND (≤1.77)	ND (≤2.02)	ND (≤0.18)
Reduction factor (log_{10})	≥4.86	≥4.60	≥6.87

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

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

Sample	Total virus titer (log_{10} $TCID_{50}$)	
	BVDV	BHV
Urokinase solution spiked with virus	5.29±0.40	6.38 ± 0.36
Pre-filtered urokinase solution	5.18±0.44	6.05 ± 0.34
Filtrate after Viresolve NFP filtration	ND[b] (≤0.69[c])	ND[b] (≤0.94[c])
Post-washed solution	ND (≤0.69)	ND (≤0.94)
Reduction factor (log_{10})	≥4.60	≥5.44

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

Process Solution I than Process Solution II upon filtration through the Viresolve NFP membrane. This is not unexpected considering that Process Solution II contained albumin as the stabilizer, which might contribute to the decreased filter life, viz. clogging of the filter. Also, high concentrations of protein can cause the membrane to become polarized. Polarization of the membrane causes loss of flux. From these filterability results, the Viresolve NFP filter was positioned at the desalted urokinase solution stage.

The feasibility test for Viresolve NFP filter has shown that there was a high recovery of product when urokinase was passed through the filter. This result indicates that this membrane has minimal protein adsorption, which makes it highly suitable for filtration of protein-containing products with high recovery efficiency. The operating time required for one batch solution of 3,500 ml to be filtered through Viresolve NFP cartridge was only 30 min. High process flow rates can provide several benefits by reducing process time, installed membrane area, product loss, and production space. Both the specific activity and the ratio of the urokinase activity in the high-molecular-mass fraction to that in the total urokinase fraction 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 urokinase.

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 filters performance. PPV was chosen as a model virus for human parvovirus 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. 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. 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, 17].

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 can not 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, pre-filters should be used before virus removal filtration to remove virus aggregates or debris that can falsely increase clearance. Pre-filtration can use sterilizing-grade (0.1 or 0.22 μm) membrane. For this study, 0.22 μm Millex-GV membrane (Millipore) was used as a pre-filter. The result presented in the present study showed that all the viruses tested were completely removed during the Viresolve NFP filtration process. This result demonstrated that the Viresolve NFP filter is effective for removing smaller size viruses of PPV, HAV, and EMCV as well as BVDV and BHV from urokinase 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.

Acknowledgments

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