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Removal and Inactivation of Hepatitis A Virus during Manufacture of Urokinase from Human Urine

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Abstract The purpose of the present study was to examine the efficacy and mechanism of the PAB (para-amino benzamidine) affinity column chromatography, Viresolve NFP virus filtration, pasteurization (60°C heat treatment for 10 h), and lyophilization steps employed in the manufacture of urokinase from human urine as regards the removal and/or inactivation of the hepatitis A virus (HAV). Samples from the relevant stages of the production process were spiked with HAV and subjected to scale-down processes mimicking the manufacture of urokinase. Samples were collected at each step, immediately titrated using a 50% tissue culture infectious dose ($TCID_{50}$), and the virus reduction factors evaluated. PAB chromatography was found to be an effective step for removing HAV with a log reduction factor of 3.24. HAV infectivity was rarely detected in the urokinase fraction, while most of the HAV infectivity was recovered in the unbound and wash fractions. HAV was completely removed during the Viresolve NFP filtration with a log reduction factor of ≥ 4.60. Pasteurization was also found to be an effective step in inactivating HAV, where the titers were reduced from an initial titer of 7.18 $\log_{10}$ $TCID_{50}$ to undetectable levels within 10 h of treatment. The log reduction factor achieved during pasteurization was ≥ 4.76. Lyophilization revealed the lowest efficacy for inactivating HAV with a log reduction factor of 1.48. The cumulative log reduction factor was ≥ 14.08. Accordingly, these results indicate that the production process for urokinase exhibited a sufficient HAV reducing capacity to achieve a high margin of virus safety.

Keywords: urokinase, hepatitis A virus, removal, inactivation, log reduction factor

INTRODUCTION

Urokinase is an enzyme with fibrinolytic activity (plasminogen activator) that is isolated from the fresh urine of healthy men. It consists of a mixture of low-molecular mass (33 kDa) and high-molecular mass (54 kDa) forms, with the high-molecular mass form being predominant. Urokinase has been reported to be effective in treating cerebral thrombosis, myocardial infarction, and arteriovenous thrombosis in the limbs and to potentiate the effect of anti-tumor drugs [1]. Even though urokinase is highly purified for clinical application, special precautions must still be taken during the production of this protein to assure against the possibility of the product transmitting infectious diseases to the recipients, as urokinase is manufactured from human urine. The most serious concern associated with the use of urokinase is the possible transmission of the hepatitis A virus (HAV) [2]. HAV is a member of the *Picornaviridae* family, which is a non-enveloped, small (25-30 nm), single-stranded RNA virus with a medium to high resistance to physico-chemical inactivation [3-6]. For this reason, the implementation of multiple viral clearance (inactivation and/or removal) steps has been highly recommended for the manufacture of urokinase [7,8].

The Green Cross Biotech Company is currently producing a high purity urokinase 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. The manufacturing process, a unique combination of process steps designed to enhance product safety, also includes virus-filtration using Viresolve NFP for virus removal and pasteurization for virus inactivation. Validation of the process for viral removal and/or inactivation can play an essential and important role in establishing the safety of biological products [9-11]. Accordingly, the current study was designed to evaluate the efficacy and mechanism of the PAB affinity chromatography, Viresolve NFP filtration, pasteurization, and lyophilization steps employed in the manufacture of urokinase as regards the removal and/or inactivation of HAV.

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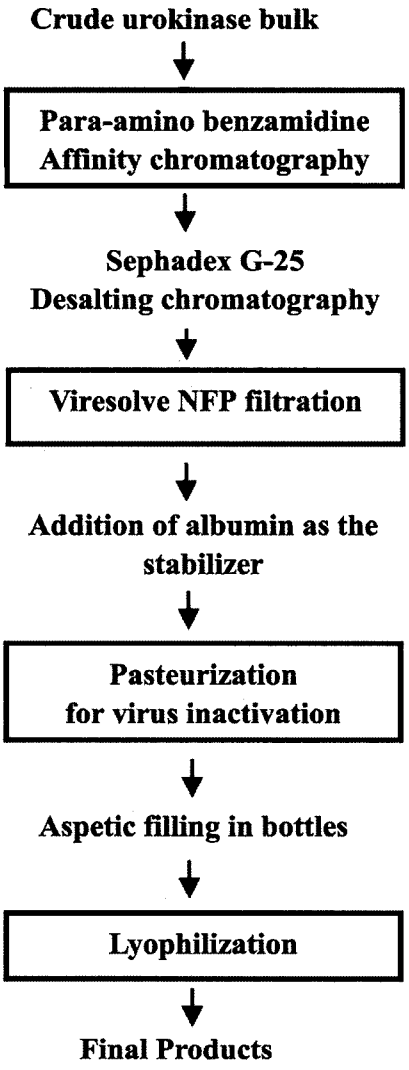

ig. 1. Flow diagram of manufacturing process for urokinase. oxes indicate the validation steps evaluated for HAV removal nd/or inactivation.

ATERIALS AND METHODS

rokinase Manufacturing Process

Urokinase for clinical use was highly purified using AB affinity column chromatography from a bulk ma- erial prepared by a successive adsorption and extrac- ion process using bentonite and calcium phosphate Fig. 1). After the PAB chromatography, the purified rokinase was desalted using Sephadex G-25 column hromatography. The desalted urokinase solution was irus-filtered using a Viresolve NFP cartridge filter. The irus-filtered urokinase solution was stabilized with .4% (w/v) albumin and pasteurized at 60 ± 0.5°C for 20 min to inactivate any contaminating viruses. The asteurized urokinase solution was placed in bottles nd lyophilized for clinical use.

Validation of Scale-down Process

The scale-down of the purification process is an essential part of process validation studies for virus removal and/or inactivation [7,8]. The scale-down of the PAB affinity column chromatography was validated by comparing the column bed-height, linear flow-rate, flow-rate-to-bed-volume ratio (*i.e.*, contact time), buffer and gel types, pH, temperature, concentration of protein and salt, and recovery of urokinase activity. A Viresolve NFP disk membrane was used to simulate the process performance of the production scale cartridges. A comparison of the pH, protein content, and recovery yield of urokinase activity in the scale-down and production filtration process was conducted to ensure that the scale-down filtration run was representative of the manufacturing process. To ensure that the scale-down pasteurization procedure was equivalent to that used in the manufacture of urokinase in the production facility, the physico-chemical properties, such as the pH, protein content, and recovery yield of urokinase activity, were compared. For a comparison of the lyophilization process, the moisture content, solubility, and urokinase activity after freeze-drying were evaluated. The lyophilization was performed using an experimental freeze dryer (VirTis, Genesis 25XL, USA). All the physico-chemical analyses were conducted according to Standard Operating Procedures (SOPs) based on the Korean Pharmacopoeia, European Pharmacopoeia, and US Pharmacopoeia.

Preparation and Titration of HAV

FRhK-4 (ATCC CRL-1688) cells were grown in a high glucose Dulbecco's modified Eagle's medium (HG DMEM) containing 2% fetal bovine serum (FBS). The cell monolayers were infected with the HAV strain HM/175/18f clone B (ATCC VR-1402) and the culture examined regularly for a cytopathic effect (cpe). When cpe became evident, the culture supernatant and cell debris were frozen, thawed, then harvested. Any cell debris was removed by centrifugation and the resultant supernatant 0.45 μm filtered, aliquoted, and frozen at −70°C. All the experiments, including the preparation and titration of HAV and virus spiking experiments, were performed in a Category III containment laboratory.

An aliquot from each sample and the appropriate control were titrated immediately upon collection in 7-fold serial dilutions ending in a quantal 50% tissue culture infectious dose ($TCID_{50}$) assay using FRhK-4 (ATCC CRL-1688) cells. The cell monolayers in 24-well culture plates were infected using at least eight 0.25 mL replicates of the appropriate dilution of the sample or positive control. The negative control wells were mock-infected using at least eight 0.25 mL replicates of the culture medium. The plates were incubated at 37°C for approximately 1 h, then the wells were fed with 1 mL of the tissue culture medium. After 14 days incubation the wells were examined for cpe. As a part of the virus

validation protocol, cytotoxicity, interference, and load titer tests were all performed. The cytotoxicity tests were performed on the 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. The interference studies were performed to determine whether the test materials exerted an inhibitory effect on the ability of the cell lines to permit detection of the virus. The load titer assay was performed to determine whether spiking the starting material with the virus would affect the virus titer.

Virus Spiking Studies

Process validation experiments were performed by spiking the starting material at each step of the process with an aliquot of the HAV stock solution that was 10% (v/v) of the total volume of the material. This was to ensure that the nature of the starting material was unaffected by the addition of the virus to the tissue culture medium. A control sample was taken after the addition of HAV to the starting material. After subjecting the sample to the processing step, the appropriate fractions were collected for an assay of the infectious virus.

To determine how the HAV partitioned during the PAB affinity column chromatography, the urokinase solution was spiked with HAV, then a sample was immediately removed for HAV titration. The remaining spiked solution was applied to the PAB affinity chromatography column to which urokinase specifically binds. The PAB column was washed to remove any unbound protein contaminants and virus. After washing the column with a washing buffer, the urokinase was eluted with an elution buffer. After eluting the urokinase, the column was washed with a high-salt buffer containing 2.0 M sodium chloride to determine how much HAV still remained bound to the column. The unbound, wash, eluate, and high-salt wash fractions were also collected. All samples were titrated immediately.

To determine the effectiveness of the Viresolve NFP filtration step in eliminating HAV, a Viresolve NFP disk membrane was used to simulate the process performance of the production scale cartridges. The urokinase solution was pre-filtered using a 0.1 μm membrane (Millex-W, Millipore). This pre-filtration was performed within 5 min prior to spiking the test solution with the virus. 18 mL of the pre-filtered urokinase solution was spiked with 2 mL of the virus stock solution. The virus-spiked urokinase solution was then pre-filtered using a 0.22 μm Millex-GV membrane (Millipore) to remove any viral aggregates, particulate, host cells, or viruses binding to proteins. Subsequently, the pre-filtrate was filtered through the disk membrane at a constant pressure of 2.0 bar. 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. Thereafter, the 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 any leaks. Aseptic pressured air was used during the filtration and the virus filtration process was conducted in a cold chamber at 2 to 8°C. All the samples generated during the filtration process were titrated immediately.

A 27 mL aliquot of the urokinase solution containing 0.4% albumin as a stabilizer was spiked with 3 mL of the HAV stock solution, then a 3 mL load sample was removed for titration. The remaining material was heated in a water bath and equilibrated to 59 ± 0.5°C. Samples were removed at different times over 600 min. An aliquot of each sample was immediately titrated.

The final step in the manufacture of urokinase is freeze-drying. As such, the final urokinase solution was spiked with the virus stock and then placed in final containers. The HAV titer was measured before and after lyophilization using an experimental freeze dryer (VirTis, Genesis 25XL, USA).

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, as previously described [12].

RESULTS AND DISCUSSION

Validation of Scale-down Process

The most essential part of process validation studies for virus removal/inactivation is proving the validity of the scale-down process. For several reasons, that include the scale of the production process and Good Manufacturing Practice (GMP) constraints regarding the introduction of viruses, it is either impossible or impractical to perform such studies on a full manufacturing scale. Therefore, the steps to be studied have to be scaled-down onto a laboratory scale [7,8]. Table 1 shows a typical comparison of some of the process parameters for the manufacturing and scale-down runs of the PAB affinity column chromatography. The reliability and reproducibility of the scale-down column to perform within the production specifications were evaluated using three validation runs. A comparison between the recovery yield and the specific activity of the eluate fractions revealed no significant difference between the manufacturing and scale-down systems.

To ensure the scale-down Viresolve NFP filtration procedure was equivalent to that used in the manufacture of urokinase in the production facility, physicochemical analyses were conducted before and after filtration (Table 2). A comparison of the recovery yields of urokinase and protein after filtration confirmed that the scale-down filtration was representative of the manufacturing run.

A comparison of the pH, protein, and urokinase activity in the manufacturing and scale-down pasteurization

Table 1. Comparison of typical parameters for manufacturing and scale-down runs of PAB affinity column chromatography process

Item	Manufacturing runs	Scale-down runs	Scale-down factor
Resin-bed dimensions (cm)	25 (diameter) 10.2 (height)	2.0 (diameter) 10.2 (height)	156
Volume of PAB gel (mL)	5,004	32	156
Volume of crude urokinase (mL)	5,500	35.3	156
Flow rate of elution (mL/min)	30	0.19	156
Specific activity of eluate after chromatography (IU/mg)	183,695 ± 11,528[a]	182,736 ± 10,436[b]	-
Recovery of urokinase (%)	94 ± 2[a]	94 ± 3[b]	-

[a] These results are the mean values of ten production batches.
[b] These results are the mean values of three independent experiments.

Table 2. Comparison of typical parameters for manufacturing, scale-down, and virus-challenge runs of Viresolve NFP filtration process

Item	Manufacturing 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
Recovery of urokinase (%)	95.28[a]	95.37[b]	-
Recovery of protein (%)	95.57[a]	95.57[b]	-

[a] These results are the mean values of ten production batches.
[b] These results are the mean values of three independent experiments.

runs confirmed that the scale-down pasteurization run was also representative of the manufacturing process (data not shown). The pH and protein content in the scale-down process remained unchanged, as observed in the production batches. The recovery yield of urokinase activity after pasteurization was 93.08 ± 0.72% for the manufacturing process and 92.96 ± 0.31% for the scale-down process.

For a comparison of the lyophilization process, key product quality measurements, such as the moisture content and recovery of urokinase activity after freeze-drying, were evaluated (Table 3). The results indicated that the scale-down lyophilization conditions were within the manufacturing specifications.

Table 3. Comparison of typical parameters for manufacturing and scale-down runs of lyophilization process

Item	Manufacturing runs	Scale-down runs
Moisture (%)	0.66± 0.24[a]	0.68 ± 0.32[b]
Recovery of urokinase after lyophilization (%)	98.4 ± 0.6[a]	98.5 ± 0.7[b]

[a] These results are the mean values of ten production batches.
[b] These results are the mean values of three independent experiments.

Partitioning of HAV through PAB Affinity Column Chromatography

Viral clearance can be achieved through routine processing and purification operations. The chromatography process physically separates virus particles from the product based on size, charge, density, binding affinity, and other differences between the virus and the product [3,14]. To evaluate the effectiveness of the PAB affinity column chromatography step in partitioning HAV, the elution profile of HAV through chromatography was assessed (Table 4). The PAB affinity column chromatography was found to be an effective step in removing HAV with a log reduction factor of 3.24. Most of the infectious virus was identified in the unbound and wash fractions. However, even though nearly all the virus was partitioned from the urokinase, HAV infectivity was still detected in the high-salt washing fraction, thereby indicating that extensive washing and cleaning of the PAB column and validation of these processes are necessary when regenerating the column.

There have already been several reports on the use of ion-exchange or monoclonal antibody chromatography to reduce HAV contamination in a number of plasma products, including the antihemophilic factor VIII and

Table 4. Removal of HAV through PAB column chromatography

Sample	Total HAV titer (Log_{10} $TCID_{50}$)	Log reduction factor
Urokinase solution spiked with HAV	7.48 ± 0.32	-
Unbound fraction	7.18 ± 0.44	-
Wash fraction	6.99 ± 0.43	-
Eluate fraction	4.24 ± 0.40	3.24
High-salt wash fraction	3.77 ± 0.51	-

These results are the mean values of three independent experiments.

Table 5. Removal of HAV through Viresolve NFP filtration

Sample	Total HAV titer ($Log_{10}TCID_{50}$)	Log reduction factor
Urokinase solution spiked with HAV	6.62 ± 0.34	-
Pre-filtered urokinase s olution	6.62 ± 0.40	-
Filtrate after Viresolve NFP filtration	ND[a] (≤ 2.02[b])	≥ 4.60
Post-washed solution	ND (≤2.02)	≥ 4.60

These results are the mean values of three independent experiments.

[a] HAV infectivity was not detected.

[b] Theoretical minimum detectable levels were used for calculation, where HAV infectivity was not detected.

IX and albumin [5,15-17], yet there are no published results on the partitioning of HAV through the chromatographic purification of urokinase.

Removal of HAV through Viresolve NFP Filtration

To evaluate the effectiveness and robustness of the Viresolve NFP filtration process in eliminating HAV, a virus validation study was performed under worst-case conditions (Table 2). The highest throughput volume-to-surface area ratio was adopted as the worst-case condition for the filtration process. The maximum working volume for the Viresolve NFP cartridge during the production process was previously set at 4,000 mL. Therefore, based on this specification, the maximum throughput volume for the scale-down was set at 11.5 mL, then a 5% surplus volume was added as the worst-case condition, making a final working volume of 12.1 mL for the virus challenge experiment. The volume of the post-wash solution was another variable in the clearance evaluation studies. Here, the worst case is the maximum wash volume, because this maximizes the filtration of the virus through the membrane. Therefore, the working volume of the post-wash solution was set at 1.2 mL. Three different lots of Viresolve NFP disk membranes were challenged with HAV (Table 5). None

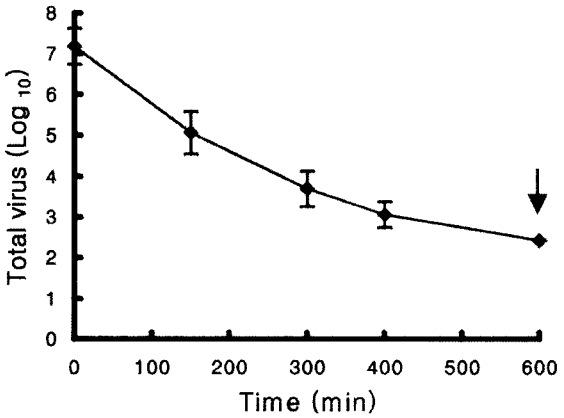

Fig. 2. Inactivation of HAV during pasteurization of urokinase at 59 ± 0.5°C and 600 min. The arrow indicates the detection limits of the quantitative assay.

of the infectious virus was detected in the filtrate of any of the 3 filter lots tested, indicating that the virus was completely removed to below detection level. The average log reduction factors achieved were ≥ 4.60.

Of the available viral clearance strategies, virus-retentive filtration is often the method of choice, being considered a robust technique and not highly susceptible to minor changes in the process conditions. Filtration for virus removal has several advantages over other conventional process steps that demonstrate a viral clearance capability [18]. Removal by size exclusion is not directly influenced by filtration conditions (*e.g.*, differential pressure, temperature, viral challenge level, *etc.*) and the characteristics of the products to be filtered (*e.g.*, viscosity, ionic strength, pH, surface tension, *etc.*). In addition, filtration is one of the least invasive processes; it does not require the inclusion of a stabilizer or other additives that are potentially toxic; it does not alter the antigenicity of the target protein or induce the formation of neoantigens; it is generally very amenable as regards inclusion into a manufacturing process. There have been several previous reports on the application of virus filters for removing possible contaminating viruses. However most of these reports have focused on the removal of viruses ranging from 120 to 35 nm in size due to the technical problems involved in making smaller sized filters [19-25].

Inactivation of HAV through Pasteurization

The principal method of inactivating virus contamination in biological products is heating in a liquid for at least 10 h at 60°C. Heat treatment, which destroys the viral envelope, has already been demonstrated to effectively inactivate viral contaminants in the preparation of many plasma-derived products [10,12,26-28]. As the worst-case conditions of pasteurization for the validation of virus inactivation, 59 ± 0.5°C and 600 min were selected as the process temperature and process time, respectively. The virus inactivation was kinetically

Table 6. Inactivation of HAV through lyophilization

Sample	Total HAV titer (Log_{10} $TCID_{50}$)	Log reduction factor
I efore lyophilization	6.70 ± 0.10	-
/ fter lyophilization	5.22 ± 0.05	1.48

hese results are the mean values of three independent experi- ents.

(valuated as a function of the process time. The HAV ' as progressively inactivated from an initial titer of 7 .18 log_{10} $TCID_{50}$ to undetectable levels within 600 min (f incubation (Fig. 2). The log reduction factor obtained ' as ≥ 4.76. Since the pasteurization experiment was (onducted under worst-case conditions, it was con- (uded that pasteurization was a robust and effective 1 easure for inactivating HAV.

] nactivation of HAV through Lyophilization

The final step in the manufacture of urokinase is ly- (philization. Lyophilization is a standard method for s abilizing labile products with limited shelf-lives in a (ilute solution. It is already known that virus infectiv- i y substantially diminished through lyophilization [5,14,15]. As such, the effect of lyophilization on the i nactivation of HAV was evaluated (Table 6). The re- s ults indicate that HAV was potentially sensitive to the l ophilization process, with an average log reduction f ictor of 1.48. The mechanism of lyophilization by ' hich viruses are inactivated is still poorly understood, l owever, it may be due to the destabilization of the (uaternary interactions between the capsid compo- 1 ents. Under normal conditions, the solvation water is i nportant in maintaining secondary protein structures,] lus it may also be involved in hydrogen bond forma- t on between different sub-units. This water is then 1 emoved during the lyophilization process, thereby dis- 1 upting the normal protein interactions.

(ONCLUSION

The cumulative virus reduction factor for a manufac- t uring process is determined from the sum of the indi- ' idual virus reduction factors based on the individual] rocess steps involved in different physicochemical 1 nethods [7,8]. In the current study, the cumulative log] IAV reduction factor achieved through different proc- (ss steps in the manufacture of urokinase was ≥ 14.08, ' hich is several magnitudes greater than the potential ' irus load of current urine pools. Accordingly, these re- s ults indicate that the production process for urokinase (xhibited a sufficient HAV reducing capacity to achieve ; high margin of safety. Regulatory guidelines recom- 1 nend incorporating multiple orthogonal methods for ' iral clearance; that is, methods that have independent (nrelated) clearance mechanisms [7,8]. Therefore, since the mechanism of HAV removal/inactivation differed in each step, it was concluded that the overall process of urokinase production was robust in reducing the HAV load. This is the first evaluation study of HAV clearance during the manufacturing process of urokinase.

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