Removal and inactivation of bovine herpes virus and murine encephalomycarditis virus by a chromatography, pasteurization, and lyophilization during the manufacture of urokinase from human urine

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Abstract

The purpose of present study was to examine the efficacy of PAB (para-amino benzamidine) affinity column chromatography, pasteurization (60℃ heat treatment for 10 h), and lyophilization steps, employed in the manufacture of urokinase from human urine, in the removal and/or inactivation of urine-born viruses. Bovine herpes virus (BHV) and Murine encephalomyocarditis virus (EMCV) were selected for this study. Samples from the relevant stages of the production process were spiked with the viruses and the amount of virus in each fraction was quantified by 50% tissue culture infectious dose ($TCID_{50}$). BHV and EMCV were effectively partitioned from urokinase during PAB chromatography with the log reduction factors of 6.71 and 5.27, respectively. Pasteurization was a robust and effective step in inactivating BHV and EMCV, of which titers were reduced from initial titers of 8.65 $\log_{10}$ $TCID_{50}$ and 7.81 $\log_{10}$ $TCID_{50}$, respectively, to undetectable levels within 1 hour of treatment. The log reduction factors achieved during lyophilization were 2.06 for BHV and 4.54 for EMCV. These results indicate that the production process for urokinase has sufficient virus reducing capacity to achieve a high margin of virus safety.

Introduction

Urokinase (UK) 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 high-molecular mass form being predominant. UK has been reported to be effective for cetebral thrombosis, myocardial infarction and arteriovenous thrombosis in the limb, and to potentiate the effect of anti-tumor drugs [1].

Though UK is 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 UK is manufactured from human urine. The possible risk associated with the use of UK is viral infection such as cytomegalovirus and hepatitis virus.

UK for clinical use is 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. The final product is sterilized by filtration and

then heated for 10 h at 60±0.5℃ to inactivate any contaminating viruses. After heat treatment, UK is filled in bottles and lyophilized for clinical use. The ability to remove and/or inactivate known and potential viral contaminants during the manufacturing process of UK has become an important parameter in assessing the safety of the products. Validation of the process for viral removal and/or inactivation can play an essential and important role in establishing the safety of urine derived products that have potential for viral contamination from the source material [2-4]. The study presented here was thus designed to evaluate the efficacy of PAB chromatography, pasteurization, and lyophilization steps employed in the manufacture of UK, in the removal and/or inactivation of viruses. Bovine herpes virus (BHV; a model virus for human herpesvirus and cytomegalovirus) and Murine encephalomyocarditis virus (EMCV; a model virus for hepatitis A virus) were selected for this study.

Materials and Methods

Validation of Process Scale-Down

Scale-down of the purification process is an essential part in performing process validation studies for virus removal and/or inactivation [2-4]. Scale-down of PAB 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 UK activity. Factor of scale-down for PAB column chromatography was about 156. To ensure that the scale-down pasteurization procedure was equivalent to that used in the manufacture of UK in the production facility, physico-chemical properties such as pH and concentrations of protein and albumin aggregate were compared. For the comparison of lyophilization process, moisture content, solubility and activity of UK after freeze-drying were evaluated. Lyophilization was performed using an experimental freeze dryer (VirTis, Genesis 25XL, USA). All the physico-chemical analyses were done according to Standard Operating Procedure (SOP) based on Korean Pharmacopoeia, British Pharmacopoeia, and US Pharmacopoeia.

Viruses and Cells

Bovine herpes virus (BHV) - enveloped, large, double-stranded DNA virus (ATCC VR-188) [16]. For propagation and titration of BHV, Madin-Derby bovine kidney (MDBK) cells (ATCC CRL-22) were used. The cells were grown in high glucose Dubbecco's modified Eagles medium (HG DMEM) contaning 10% FBS plus non-essential amino acids (NEAA).

Murine encephalomyocarditis virus (EMCV) - non-enveloped, small, single-stranded RNA virus (ATCC VR-129B) with medium to high resistance to physico-chemical reagents [18]. EMCV provides a severe test for validation of the process in the removal and/or inactivation of viruses. EMCV belongs to the Picornaviridae family, which also includes hepatitis A virus. For propagation and titration of EMCV, Vero C1008 cells (ATCC CRL-1586) were used. The cells were grown in HG DMEM containing 10% FBS plus NEAA.

Titration of Viruses

An aliquot from each sample and the appropriate control were titrated immediately upon collection in 7-fold serial dilutions to end point using a quantal 50% tissue culture infectious dose ($TCID_{50}$) assay [15]. Indicator cell monolayers in a 24-well culture plates were infected using at least eight 0.25ml replicates of the appropriate dilution of sample or positive control. Negative control wells were mock-infected using at least eight 0.25ml replicates of culture medium. The plates were incubated at 37℃ for approximately 1 hour, and the wells were fed with 1ml of tissue culture medium. As a part of virus validation protocol, cytotoxicity, interference and load titer tests were performed.

Calculation of Virus Reduction Factors

The virus reduction factor for an individual purification or inactivation step is 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 [3]. 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.

Results and Discussion

Validation of scale-down processes

All the parameters obtained for scale-down process were within the manufacturing specifications (data not shown).

Partitioning of BHV and EMCV during PAB affinity chromatography

To evaluate the effectiveness of the PAB chromatography step in eliminating BHV and EMCV, the elution profile of viruses during PAB chromatography was assessed. A bulk UK solution was spiked with viruses and then a sample was immediately removed for titration. The remaining material was applied to the PAB column and the unbound, wash, eluate, and high salt wash fractions were collected. All samples were neutralized to pH 6.5 – 7.5 and an aliquot of each sample was titrated immediately. The results in Table 1 indicate that the PAB chromatography was an effective step in removing BHV and EMCV. The average log reduction factors were 6.71 for BHV and 5.27 for EMCV.

Inactivation of BHV and EMCV during pasteurization

The effectiveness of 60℃ heat treatment for 10 h in inactivating viruses was determined. BHV was completely inactivated from an initial titer of 8.65 $\log_{10}$ $TCID_{50}$ to undetectable levels within 1 h of incubation. The log reduction factor obtained was ≥6.76. EMCV was completely inactivated from an initial titer of 7.81 $\log_{10}$ $TCID_{50}$ to undetectable levels within 1 h of incubation. The log reduction factor obtained was ≥5.92.

Inactivation of BHV and EMCV during lyophilization

The final step in the manufacture of the highly purified UK is freeze-drying. Virus stock was spiked to the final UK concentrate solution and then the virus-spiked samples were distributed in final containers. The titers of viruses were measured before and after lyophilization. The results in Table 2 indicate that BHV and EMCV were potentially sensitive to lyophilization, with log reduction factors of were 2.06 for BHV and 4.54 for EMCV.

Conclusion

The cumulative log reduction factors achieved, ≥15.53 for BHV and ≥17.73 for EMCV, were by several magnitudes greater than the potential virus load of current human urine pools. These results indicate that the production process for UK has sufficient virus reducing capacity to achieve a high margin of virus safety.

Table 1. Partitioning of BHV and EMCV during PAB affinity chromatography

Sample	Total virus titer (Log_{10} $TCID_{50}$)	
	BHV	EMCV
Starting material spiked with virus	9.60	8.23
Unbound fraction	9.06	7.95
Wash 1 fraction	8.90	8.29
Wash II fraction	6.69	6.31
Eluate fraction	3.10	2.27
High salt wash fraction	2.89	2.96
Log reduction factor	6.71	5.27

Table 2. Inactivation of BHV and EMCV during lyophilization

Sample	Total virus titer (Log_{10} $TCID_{50}$)	
	BHV	EMCV
Before lyophilization	9.24	8.29
After lyophilization	7.18	3.75
Log reduction factor	2.06	4.54

References

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