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Removal and Inactivation of Human Immunodeficiency Virus (HIV-1) by Cold Ethanol Fractionation and Pasteurization during the Manufacturing of Albumin and Immunoglobulins from Human Plasma

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Abstract Viral safety is a prerequisite for manufacturing clinical albumin and immunoglobulins from human plasma pools. This study was designed to evaluate the efficacy of cold ethanol fractionation and pasteurization (60°C heat treatment for 10 h) for the removal/inactivation of human immunodeficiency virus type 1 (HIV-1) during the manufacturing of albumin and immunoglobulins. Samples from the relevant stages of the production process were spiked with HIV-1, and the amount of virus in each fraction was quantified by the 50% tissue culture infectious dose ($TCID_{50}$). Both fraction IV fractionation and pasteurization steps during albumin processing were robust and effective in inactivating HIV-1, titers of which were reduced from an initial 8.5 $\log_{10}$ $TCID_{50}$ to undetectable levels. The log reduction factors achieved were $\geq$ 4.5 and $\geq$ 6.5, respectively. In addition, fraction III fractionation and pasteurization during immunoglobulins processing were robust and effective in eliminating HIV-1. HIV-1 titers were reduced from an initial 7.3 $\log_{10}$ $TCID_{50}$ to undetectable levels. The log reduction factors achieved in this case were $\geq$ 4.9 and $\geq$ 5.3, respectively. These results indicate that the process investigated for the production of albumin and immunoglobulins have sufficient HIV-1 reducing capacity to achieve a high margin of safety.

Keywords: albumin, immunoglobulins, cold ethanol fractionation, pasteurization, human immunodeficiency virus, log reduction value

INTRODUCTION

Human plasma, the liquid portion of blood, contains many dissolved components. These are primarily proteins which are important in the humoral immune and coagulation systems. The immune proteins are predominantly immunoglobulins and antibodies, which react specifically with antigens. Coagulation proteins contribute to a series of enzyme-substrate reactions and the platelet function that are responsible for maintaining hemostasis. Many important therapeutic products such as albumin, immunoglobulins, and coagulation factors, are purified from plasma. Although the therapeutic use of blood transfusion goes back to the turn of the century, the first significant component fractionation of plasma protein was achieved in early 1940 [1]. This separation method is often referred as cold ethanol fractionation, because the ethanol is used as a precipitating reagent at subfreezing temperatures. Stepwise changes of pH, ethanol concentration, ionic strength, protein concentration, and temperature enable fractional precipitation of major plasma components.

Because plasma-derived proteins are manufactured from human plasma, special precautions must be taken during the production of these proteins to guard against the possibility of the products transmitting infectious diseases to recipients [2,3]. For a long time the major risk associated with the use of blood products was viral infection, such as from the Human immunodeficiency virus (HIV), Hepatitis A, B, C, and G, and the Human T-cell lymphotropic viruses (HTLV) I and II [4-6]. Recently transmissible spongiform encephalopathies (TSE's) have highlighted the risks involved [7].

Human albumin solution and immunoglobulins manufactured using the cold ethanol fractionation and pasteurization procedures have an excellent viral safety record based on 50 years of clinical use [8,9]. This safety record with respect to viral transmission is attributed to a combination of several factors: (i) careful selection of donors, (ii) careful screening of units donated for known infectious viruses, (iii) viral removal and inactivation by the methods used for manufacture, and (iv) the testing of products and intermediates for viral markers. Although these plasma products have proven to be safe, it is now essential that the potency of various steps of the manufacturing process, designed to

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remove/inactivate viral infectivity, be evaluated. Validation of the process of viral inactivation/removal can play an essential and important role in establishing the safety of biological products, especially when there is a high potential for the source material to be contaminated with a virus known to be pathogenic to human [10-14].

The study presented here was designed to evaluate the efficacy of the cold ethanol fractionation and pasteurization steps for the removal/inactivation of human immunodeficiency virus type 1 (HIV-1) during the manufacture of albumin and immunoglobulins from human plasma. HIV is a member of the lentivirus family of retroviruses, members of which are enveloped, medium-sized, single-stranded, RNA viruses with a low resistance to physico-chemical inactivation. HIV is considered to be of concern when the product is derived from material of human origin, particularly blood products. The use of this virus is mandatory when performing a virus validation on human blood and plasma products [12,13].

MATERIALS AND METHODS

Plasma and Manufacturing Process for Albumin and Immunoglobulins

The plasma for these experiments was purchased from the Korean Red Cross. Samples for virus inactivation and partitioning studies were prepared from source plasma from the regular manufacturing process. The source plasma was negative for hepatitis B surface antigen, anti-HIV-1-antibody, anti-HCV-antibody, and hepatitis A RNA by polymerized chain reaction. Albumin or immunoglobulins were prepared by cold ethanol fractionation according to the Cohn-Oncley procedures [1,15]. As shown in Fig. 1, human plasma frozen below –18°C was thawed. After the insoluble protein precipitate (Cryopaste) was separated, the cryo-poor plasma protein components were fractionated by stepwise increases of the ethanol concentration and decreases of pH, according to the Cohn-Oncley method. Dissolved fraction V for albumin was ultrafiltered to remove the residual ethanol, and the resulting bulk solution was heated at 60°C for 10 h to remove unwanted proteins and sterile-filtered using a 0.2 μm filter (Millipore). The final solution was filled into bottles and pasteurized at 60°C for 10 h for virus inactivation. Fraction I+II+III paste was washed to remove the residual proteins remaining in the mother liquor of the paste. The washed fraction I+II+III which is designated as Fraction I+II+IIIw was further fractionated. The dissolved fraction II for immunoglobulins was ultrafiltered to remove the residual ethanol, and the resulting bulk solution pasteurized at 60°C for 10 h for virus inactivation. The pasteurized solution was further processed to intravenous immunoglobulins or gamma globulins.

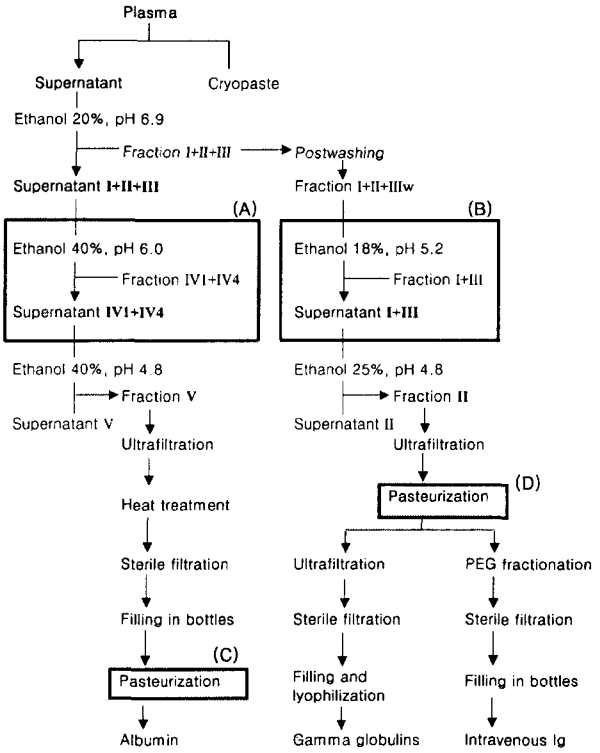

Fig. 1. Flow diagram of plasma fractionation and the manufacturing processes for albumin and immunoglobulins. Boxes indicate the validation steps evaluated for HIV-1 removal/inactivation; (A) fraction IV fractionation, (B) fraction III fractionation, (C) pasteurization for albumin, (D) pasteurization for immunoglobulins.

Validation of Process Scale-Down

Scaling-down the production process is an essential part of performing process validation studies for virus removal/inactivation [11-14]. To ensure that the performance of the scale-down process of cold ethanol fractionation was representative of the production situation, physico-chemical properties such as pH, temperature, and ethanol concentration were compared. To ensure that the scale-down pasteurization procedure was equivalent to that used in the manufacture of albumin (20%) or immunoglobulins in the production facility, physico-chemical properties such as pH and the concentrations of protein and stabilizer were compared.

All the physico-chemical analyses were performed according to the Standard Operating Procedure (SOP) based on the Korean Pharmacopoeia, British Pharmacopoeia, and US Pharmacopoeia.

Preparation and Titration of HIV-1

Human H9 cells (ATCC HTB-176) were grown in RPMI 1640 medium containing 10% fetal bovine serum (FBS) and L-glutamine (10% RPMI). The cells were infected by mixing with H9 cells chronically infected

with the RF strain of HIV-1 (Medical Research Council, AIDS Directed program no. 104), and the culture was passaged and examined regularly for cytopathic effect (cpe) and syncytial formation. At the peak of the syncytiation, the supernatant was harvested, clarified by centrifugation, 0.45 μm filtered, aliquoted and frozen at –70°C.

An aliquot from each sample and the appropriate control were titrated immediately upon collection in 7-fold serial dilutions to the end point using a quantal 50% tissue culture infectious dose ($TCID_{50}$) assay [16]. Suspensions of C8166 cells (Medical Research Council, AIDS Directed program No. 013) in microtiter 96-well plates were infected using at least eight 0.1 mL replicates of the appropriate dilution of sample or positive control of HIV-1. Negative control wells were mock-infected using at least eight 0.1 mL replicates of culture medium. The plates were incubated at 37°C and each well fed with the tissue culture medium, as appropriate. Approximately 14-21 days later the wells were examined for cpe. As a 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 exerted an inhibitory effect on the ability of the cell lines to permit detection of the virus. A load titer assay was performed to determine when spiking the virus into the starting material resulted in a loss in virus titer.

HIV-1 Removal or Inactivation during Cold Ethanol Fractionation

Preliminary studies showed that HIV-1 was very labile to the conditions of fraction IV fractionation of which ethanol concentration was 40%. Therefore, the inactivation of HIV-1 at this condition was analyzed kinetically in order to evaluate the effectiveness of the fraction IV fractionation step in inactivating HIV-1 (Fig. 1A). Fraction IV4-2 was prepared by adding 34.7 mL of 95% ethanol to 80 mL of fraction IV4-1, while maintaining the temperature at –5.5°C, over a 7 h period. A 50 mL aliquot of fraction IV4-2 suspension, at –5.5°C, was then spiked with 5 mL of HIV-1, and a 5 mL sample was removed immediately. The remaining material was placed in a cooling bath (Polyscience, Warrington, PA, USA) at –5.5°C and further samples were collected after 10 min, 30 min, 1 h, and 2 h. All the samples were 2 fold-diluted in 10% RPMI on collection to prevent further inactivation of HIV-1, and titrated immediately.

The partitioning profile of HIV-1 after fraction III fractionation was assessed in order to evaluate the efficacy of the fraction III fractionation process in eliminating HIV-1 (Fig. 1B). Ultracentrifuged HIV-1 was dissolved with fraction I+II+III-w suspension and 9 mL of the HIV-1 solution so obtained was added to 96 mL of the fraction I+II+III-w suspension. An aliquot of the HIV-1 spiked solution was taken to determine the viral titer in the spiked starting material. The HIV-1 spiked solution was continuously supplemented with 95% ethanol, at a temperature of -20°C, to a final concentration of 18%, while maintaining the temperature at –5.5°C, over a 20 h period. The material was then filtered at a pressure of 1.0-1.5 kg/cm^2 using 0.2 μm Supra-80 membranes (SeitzSchenk, Waldstetten, Germany). The resulting supernatant was collected and the paste I+III fraction was resuspended in tissue culture medium. Both samples were titrated immediately to quantify infectious HIV-1.

HIV-1 Inactivation by Pasteurization

A 60 mL aliquot of the final 20% albumin solution containing stabilizers (13.3 mg sodium caprylacid and 19.7 mg acetyl tryptophan per 1 g albumin) was spiked with 6 mL of HIV-1 and a 6 mL load sample removed for titration. The remaining material was heated in a water bath and equilibrated to 59 ± 1°C (Fig. 1C). Samples were taken at different times over 10 h. An aliquot of each sample was titrated immediately.

The dissolved fraction II solution was dialyzed against water to remove the residual ethanol, and a 35 mL aliquot of the solution was then spiked with 3.5 mL of HIV-1 and sorbitol added up to 33% (w/v). After adjusting the pH to 5.0, the resulting solution was pasteurized at 60 ± 0.5°C for 10 h (Fig. 1D). Samples were removed at different times. An aliquot of each sample was titrated immediately.

Calculation of Virus Reduction Factor

The virus reduction factor for an individual purification or inactivation step was defined as $\log_{10}$ of the ratio of the virus load in the spiked starting material divided by the virus load in the post process material [13]. The formula used took into account both the titers and volumes of the materials before and after the processing step. Reduction factors were normally expressed on a logarithmic scale.

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

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

Validation of Scale-down Process

To ensure that the performance of the scale-down cold ethanol fractionation process was representative of that used for production, several parameters of the scale-down process were compared with those of the manufacturing process. Each scale-down process was

Table 1. Comparison of the physicochemical parameters between untreated and pasteurized 20% albumin obtained from the scale-down and production pasteurization processes

Parameter	Sample	Scale-down (n = 5)	Production (n = 10)
pH	Untreated	7.02 ± 0.05	7.02 ± 0.06
	Pasteurized	7.02 ± 0.04	7.02 ± 0.05
Protein concentration (%, w/v)	Untreated	20.26 ± 0.21	20.30 ± 0.15
	Pasteurized	20.24 ± 0.18	20.26 ± 0.14
Purity of albumin (%)	Untreated	99.02 ± 0.34	99.11 ± 0.29
	Pasteurized	98.99 ± 0.28	98.92 ± 0.24
Protein aggregation	Untreated	0	0
	Pasteurized	0.32 ± 0.05	0.38 ± 0.06

performed five times. The ethanol concentration, pH, and temperature during the scale-down fraction IV fractionation process were 39.8 ± 0.4% (w/v), 6.0 ± 0.1, and −5.5°C, respectively, which were within the manufacturing specifications.

The ethanol concentration, pH, and temperature during the scale-down fraction III fractionation process were 18.1 ± 0.3% (w/v), 5.2 ± 0.1, and −5.5 °C, respectively, which again were within the manufacturing specifications.

To ensure that the scale-down pasteurization procedure was equivalent to that used in the manufacture of 20% albumin, physicochemical analyses of samples before and after pasteurization were conducted. Comparisons of pH, protein and aggregate content for the scale-down and production pasteurization process are presented in Table 1. The pH and protein content of the scale-down process were similar to those observed in production batches, and the aggregate content increased on pasteurization in 20% albumin to levels routinely observed in production. The results demonstrate that the scale-down pasteurization run was representative of the manufacturing process.

For validation of the scale-down pasteurization procedure for immunoglobulins, the pH and concentration of stabilizer (sorbitol) were determined. The pH and concentration of sorbitol were 5.1 ± 0.2 and 32.9 ± 0.3 (w/v), respectively, which again were within normal production limits.

Inactivation of HIV-1 during Fraction IV Fractionation Process

The time course of inactivation of HIV-1 under the conditions of fraction IV fractionation (ethanol 40%, pH 6.0, -5.5°C) was analyzed (Fig. 2). HIV-1 was completely inactivated from an initial titer of 8.5 $\log_{10}$ $TCID_{50}$ to undetectable levels within 2 h, and rapid inactivation of HIV-1 was achieved during the first ten minutes of treatment. Residual infectivity was detected after 1 h but was absent after 2 h. The log reduction factor obtained was ≥ 4.5.

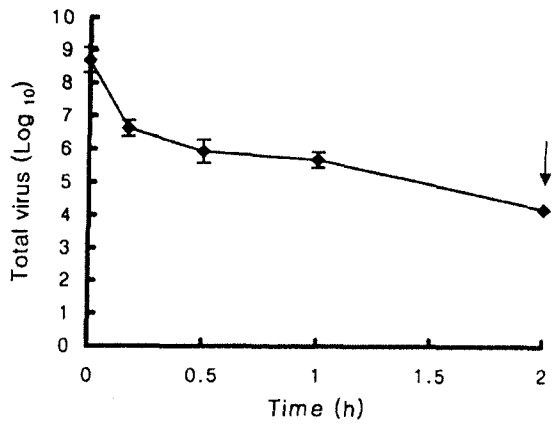

Fig. 2. Kinetics of inactivation of HIV-1 during fraction IV fractionation. The arrows indicate the detection limits of the quantitative assay.

Table 2. Reduction of HIV-1 during fraction III fractionation

Exp. No.	Sample	Total HIV-1 titer (Log_{10} $TCID_{50}$)	Reduction factor (Log_{10})
1	Spiked HIV-1	7.3	–
	Supernatant	2.4*	≥4.9
	Paste	3.5*	–
2	Spiked HIV-1	7.3	–
	Supernatant	2.4*	≥4.9
	Paste	3.5*	–

*No infectious HIV-1 was detected. These values were calculated using a theoretical minimal detection level of infectious virus with a 98% confidence level.

Reduction of HIV-1 during Fraction III Fractionation Process

The partitioning profile of HIV-1 after fraction III fractionation was assessed in order to evaluate the efficacy of the fraction III fractionation process in eliminating HIV-1. After separation of the precipitates, the HIV-1 titers in the resulting supernatant and the paste I+III fraction were analyzed (Table 2). No infectious virus was detected in the supernatant, indicating the complete removal of HIV-1 during fraction III fractionation with the log reduction factor of ≥4.9. In addition, it was also found that no infectious virus was present in the paste. These results indicate that all the spiked HIV-1 was inactivated during fraction III fractionation by the added ethanol, and thus that the mechanism of HIV-1 reduction was inactivation rather than partitioning, under this experimental condition.

HIV-1 Inactivation during Pasteurization

The effectiveness of pasteurization in inactivating HIV-1 during the manufacturing of albumin and im-

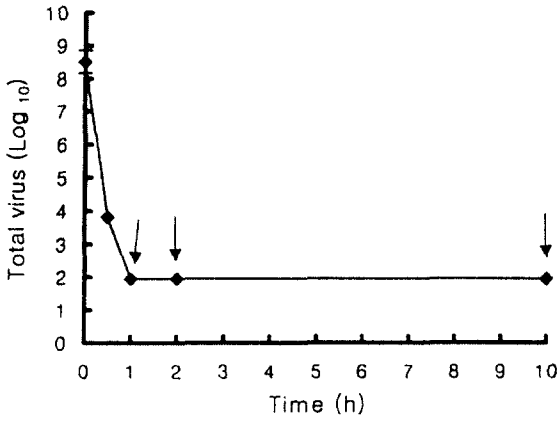

Fig. 3. Inactivation of HIV-1 during pasteurization of albumin at 59°C for 10 h. The arrows indicate the detection limits of the quantitative assay.

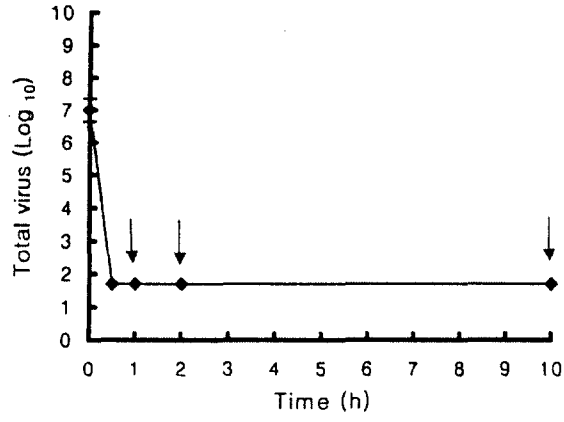

Fig. 4. Inactivation of HIV-1 during pasteurization of immunoglobulins at 60°C for 10 h. The arrows indicate the detection limits of the quantitative assay.

munoglobulins was also determined. HIV-1 spiked to 20% albumin solution was completely inactivated from an initial titer of 8.5 $\log_{10}$ $TCID_{50}$ to undetectable levels within 1 h of incubation at 59°C (Fig. 3), with the log reduction factor of ≥6.5.

HIV-1 spiked to the dissolved fraction II solution containing 33% sorbitol as a stabilizer was completely inactivated from an initial titer of 7.0 $\log_{10}$ $TCID_{50}$ to undetectable levels within 0.5 h of incubation at 60°C (Fig. 4), with the log reduction factor of ≥5.3.

DISCUSSION

The most essential part of performing process validation studies for virus removal/inactivation is to prove 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 these studies on the full manufacturing scale. Therefore, the steps to be studied are scaled-down to laboratory scale [11-14]. All the parameters chosen to confirm the validity of the scale-down process in this study were within the manufacturing specifications, which indicated that the scale-down processes were well designed to mimic as closely as possible the full-scale manufacturing processes.

Ethanol is known to be both bactericidal and viricidal and is the principal precipitation agent in cold ethanol fractionation used in the manufacture of plasma products. The highest concentration of ethanol used in the fractionation is 40%, and in other stages it is 8-25%. In addition to the viricidal effect of ethanol, the partitioning of viruses during fractionation is important in determining the effectiveness of this process for the production of safe plasma products [17]. The fraction IV fractionation step was very robust and effective in inactivating HIV-1. The inactivation kinetics showed that the time needed for the complete inactivation of HIV-1 in this experimental condition was 2 h. In consideration of the fact that fraction IV1+IV4 paste is separated from the supernatant IV1+IV4 by filtration after 8 h incubation in fraction IV fractionation conditions (40% ethanol, pH 6.0) during the manufacturing of albumin, it is likely that the extent of HIV-1 inactivation is independent of the initial viral load. The fraction III fractionation step (18% ethanol, pH 5.2) was also effective in eliminating HIV-1. The mechanism of reduction in supernatant of fraction III was inactivation rather than partitioning, indicating that HIV-1 is very vulnerable to ethanol. These experimental findings support the results of epidemiological studies, which showed that no HIV transmission has resulted from the administration of albumin or immunoglobulin manufactured by cold ethanol fractionation [8,9].

The principal method for the inactivation of virus contamination in plasma products involves heating in a liquid for at least 10 h at 60°C. Heat treatment that destroys the viral envelope has been demonstrated by a number of investigators to effectively inactivate HIV contaminants in the preparation of many plasma-derived products [18,19]. The present results indicate that pasteurization at 60°C resulted in the complete inactivation of HIV-1 (≥ 6.5 $\log_{10}$ reduction to below the detection level for albumin process and ≥ 5.3 $\log_{10}$ reduction to below the detection level for immunoglobulins process). These results are consistent with those of other investigations that showed pasteurization to be an effective means of providing an extra measure of viral safety in plasma derivatives [19-22]. The inactivation kinetics of HIV-1 showed that the time needed for the complete inactivation of HIV-1 in this experimental condition was 1 h for albumin and 0.5 h for immunoglobulins, respectively. From these results it was concluded that pasteurization is a robust and effective way of eliminating HIV-1. Since viral load was reduced to undetectable levels within the first hour of the 10 h of

incubation in these experiments, it was further concluded that the extent of viral inactivation is independent of the initial viral load.

The results of this study indicate that cold ethanol fractionation and pasteurization during the manufacturing of albumin and immunoglobulins result in complete removal and inactivation of spiked HIV-1. The present findings suggest that the excellent safety record of albumin and immunoglobulins is explained by the effectiveness and robustness of these two processes.

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