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Partitioning and Inactivation of Viruses by Cold Ethanol Fractionation and Pasteurization during Manufacture of Albumin from Human Plasma

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Abstract The purpose of the present study was to examine the efficacy and mechanism of the fraction IV cold ethanol fractionation and pasteurization (60°C heat treatment for 10 h) steps, involved in the manufacture of albumin from human plasma, in the removal and/or inactivation of blood-born viruses. A variety of experimental model viruses for human pathogenic viruses, including the Bovine viral diarrhoea virus (BVDV), Bovine herpes virus (BHV), Murine encephalomyocarditis virus (EMCV), and Porcine parvovirus (PPV), 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 then quantified using a 50% tissue culture infectious dose ($TCID_{50}$). The mechanism of reduction for the enveloped viruses (BHV and BVDV) during fraction IV fractionation was inactivation rather than partitioning, however, it was partitioning in the case of the non-enveloped viruses (EMCV and PPV). The log reduction factors achieved during fraction IV fractionation were ≥6.9 for BHV, ≥5.2 for BVDV, 4.9 for EMC, and 4.0 for PPV. Pasteurization was found to be a robust and effective step in inactivating the enveloped viruses as well as EMCV. The log reduction factors achieved during pasteurization were ≥7.0 for BHV, ≥6.1 for BVDV, ≥6.3 for EMCV, and 1.7 for PPV. These results indicate that the production process for albumin has sufficient virus-reducing capacity to achieve a high margin of virus safety.

Key words: Human albumin, cold ethanol fractionation, pasteurization, virus removal and inactivation

Human serum plasma contains various therapeutic protein components. Recovery and purification of clinically useful components, such as albumin, immunoglobulins, and coagulation factors, from plasma has been a challenging subject for over a half century and the trend continues [3]. Although the therapeutic use of blood transfusion goes back almost 100 years, the first significant component fractionation of plasma protein was achieved in the early 1940s [4]. This separation method is often referred to cold ethanol fractionation since ethanol is used as the precipitating reagent at a subfreezing temperature. A stepwise change of the pH, ethanol concentration, ionic strength, protein concentration, and temperature enables the fractional precipitation of the major plasma components.

Because plasma-derived medicinal products are manufactured from human plasma, special precautions must be taken during the production of these proteins to assure against the possibility of the products transmitting infectious diseases to the recipients [11, 20]. For a long time, the major risk associated with the use of blood products has been viral infections, such as Hepatitis A, B, C, and G, Human immunodeficiency virus (HIV), Human T-cell lymphotropic viruses (HTLV) I and II, and parvoviruses [1, 5, 28].

Albumin solutions for clinical use are manufactured from fraction V paste prepared by the cold ethanol fractionation of large quantities of pooled plasma. The final product is sterilized by filtration and then heated for 10 h at 60±0.5°C to inactivate any contaminating viruses. The ability to remove and/or inactivate known and potential viral contaminants during the manufacturing process of plasma derivatives 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 plasma-derived products that have high potential for viral contamination from the source material [12]. Accordingly, this study was designed to evaluate the efficacy and mechanism of the fraction IV cold ethanol fractionation and pasteurization steps, employed in the manufacture of albumin from human plasma, for the removal and/or inactivation of viruses. In this study, the Bovine viral diarrhoea virus (BVDV; a model virus for hepatitis C or hepatitis G viruses) and Bovine herpes virus (BHV; a model virus for the human herpesvirus, such as

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HHV-6, HHV-7, HHV-8, Epstein Barr virus, or HSV-1) were selected as models of lipid enveloped viruses, whereas the Murine encephalomyocarditis virus (EMCV; a model virus for hepatitis A virus) and Porcine parvovirus (PPV; a model virus for human parvovirus) were chosen as examples of non-lipid enveloped viruses.

MATERIALS AND METHODS

Plasma and Albumin Manufacturing Process

The plasma for these experiments was purchased from the Korean Red Cross. The samples for the virus inactivation and partitioning studies were prepared from the source plasma using the regular manufacturing process. The source plasma indicated negative for the hepatitis B surface antigen, anti-HIV-1-antibody, anti-HCV-antibody, and hepatitis A RNA based on a polymerized chain reaction. The albumin was prepared from the source plasma using cold ethanol fractionation according to the Cohn-Oncley procedures [4, 23]. As shown in Fig. 1, after thawing the frozen human plasma, the insoluble protein precipitates (Cryoprecipitate) were harvested and used as the raw material for manufacturing antihemophilic Factor VIII. The plasma protein components were then fractionated by a stepwise increase of the ethanol concentration plus a decrease in the pH according to the Cohn-Oncley method. The dissolved fraction V was concentrated by ultrafiltration, then the resulting bulk solution was heated at 60°C for 10 h to remove any unwanted proteins and sterile-filterd using a 0.2 μm filter (Millipore). The final solution was filled in bottles and heated at 60°C for 10 h for virus inactivation.

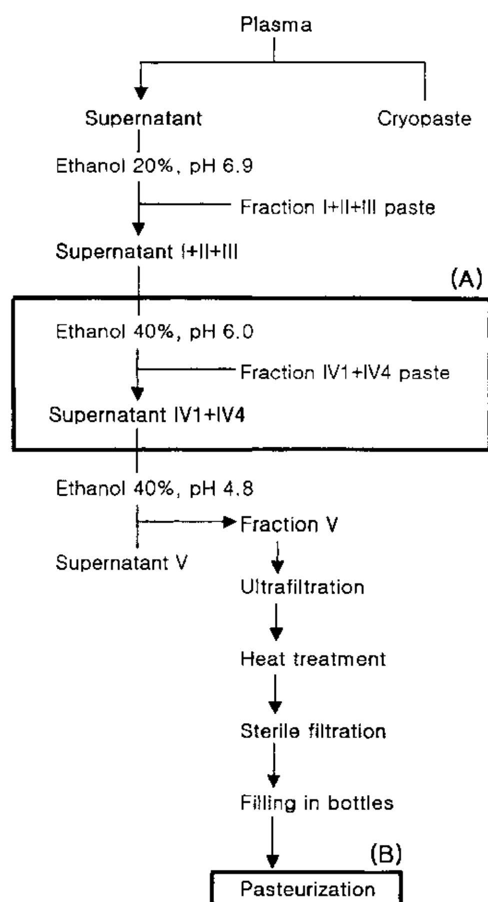


Fig. 1. Flow diagram of plasma fractionation and manufacturing process for albumin.
The boxes indicate the validation steps evaluated for virus removal/inactivation; (A) fraction IV fractionation, (B) pasteurization for albumin.

Validation of Process Scale-Down

The scale-down of the purification process is an essential part of performing process validation studies for virus removal and/or inactivation [12, 15]. To ensure that the performance of the scale-down process of fraction IV precipitation was representative of the production scale, the physico-chemical properties, such as the pH, temperature, and ethanol concentration, were compared. To ensure that the scale-down pasteurization procedure was equivalent to that used in the manufacture of 20% albumin in a production facility, the physico-chemical properties, such as the pH and concentrations of protein and albumin aggregate, were also compared. All the physico-chemical analyses were done according to the Standard Operating Procedure (SOP) based on the Korean Pharmacopoeia, British Pharmacopoeia, and US Pharmacopoeia.

Viruses and Cells

The viruses used in this study were selected to demonstrate the inactivation and/or removal of viruses with a range of biophysical and structural features which may reflect the presence of any unknown or unidentified contaminants in the starting material and displaying a significant resistance to physical or chemical agents [12]. The viruses selected based on this rationale were as follows.

Bovine herpes virus (BHV) - enveloped, large, double-stranded DNA virus (ATCC VR-188) [16]. Herpesviruses can remain as latent infections within lymphoid cells, and several herpesviruses, such as HSV-1, HSV-2, HCMV, HHV-6, HHV-7 and HHV-8 are potentially transmissible by blood and plasma. Therefore, BHV was selected as it is representative of this class of virus, can be obtained in sufficient titer for validation studies, and is not neutralized by the presence of antibodies in the plasma. For the propagation and titration of BHV, Madin-Derby bovine kidney (MDBK) cells (ATCC CRL-22) were used, which were grown in a high glucose Dubbecco's modified Eagles medium (HG DMEM) containing 1% FBS plus non-essential amino acids (NEAA).

Bovine viral diarrhoea virus (BVDV) - enveloped, medium-sized, single-stranded RNA virus (ATCC VR-534) with

a medium resistance to physico-chemical reagents [16]. BVDV belongs to the Flaviviridae family, which also includes the hepatitis C virus [8, 24]. BVDV is therefore a suitable model virus where hepatitis C is of concern, particularly, in products derived from human blood. It is also a model for the hepatitis G virus. For the propagation and titration of BVDV, bovine turbinate (BT) cells (ATCC CRL-1390) were used, which were grown in HG DMEM containing 1% FBS plus NEAA.

Murine encephalomyocarditis virus (EMCV) - non-enveloped, small, single-stranded RNA virus (ATCC VR-129B) with a medium to high resistance to physico-chemical reagents [16]. EMCV provides a severe test for the validation of a process as regards the removal and/or inactivation of viruses. EMCV belongs to the Picornaviridae family, which also includes the hepatitis A virus [14]. For the propagation and titration of EMCV, Vero C1008 cells (ATCC CRL-1586) were used, which were grown in HG DMEM containing 1% FBS plus NEAA.

Porcine parvovirus (PPV) - non-enveloped, small, single-stranded DNA virus (ATCC VR-742) [16]. This virus has a high resistance to a range of physico-chemical reagents and is a known contaminant of porcine blood. It therefore provides a severe test for the validation of a process in regards to the removal and/or inactivation of viruses. For the propagation and titration of PPV, minipig kidney (MPK) cells (ATCC CCL-166) were used, which were grown in HG DMEM containing FBS.

Titration of Viruses

An aliquot from each sample and an 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 [13]. Indicator cell monolayers in 24-well culture plates were then infected using at least eight 0.25 ml replicates of the appropriate dilution of each sample or the positive control. Negative control wells were mock-infected using at least eight 0.25 ml replicates of the culture medium. The plates were then incubated at 37°C for approximately 1 h, and the wells were fed with 1ml of the tissue culture medium.

As a part of the virus validation protocol, cytotoxicity, interference, and load titer tests were also performed. The cytotoxicity tests were performed on those 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 tests were performed to determine whether the starting materials for the fraction IV fractionation and pasteurization exerted an inhibitory effect on the ability of the cell lines to permit the detection of the virus. The load titer assays were performed to determine where spiking the virus into the starting material resulted in a loss in the virus titer.

Virus Partitioning by Fraction IV Fractionation

The partitioning profile of the viruses after the fraction IV fractionation was assessed in order to evaluate the efficacy of the fraction IV fractionation process in eliminating viruses. 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. An 80 ml aliquot of the fraction IV4-2 suspension, at -5.5°C, was then spiked with 8 ml of virus. An 8 ml aliquot of the sample was then immediately removed and diluted 1:9 in 1% DMEM. The remaining material was maintained at -5.5°C over a period of 8 h and then filtered at a pressure of 1.0-1.5 kg/cm^2 using 0.2 µm Supra-80 membranes (Seitz-Schenk, German). The resulting filtrate and supernatant fractions were collected and the fraction IV paste was resuspended in the tissue culture medium with an equivalent volume to that of the supernatant. All samples were neutralized to pH 6.5-7.5 upon collection where required, and an aliquot from each sample was immediately titrated.

Virus Inactivation during Fraction IV Fractionation

Enveloped viruses, such as BHV and BVDV, are labile to the conditions of fraction IV fractionation. Therefore, the inactivation of enveloped viruses was analyzed kinetically in order to evaluate the effectiveness of the fraction IV fractionation step in inactivating enveloped viruses. Five ml of each virus stock was spiked in a 50 ml aliquot of fraction IV4-2 and incubated at -5.5°C. Samples were removed at different times and immediately titrated.

Virus Inactivation during Pasteurization

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

Calculation of Virus Reduction Factors

The virus reduction factor for each 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 [12, 15]. The formula then takes into account 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=the reduction factor for a given stage, v^{I}=the volume of the input material, aI=the titer of the virus in the input material, v^{II}=the volume of the retained output material, aII=the titer of the virus in the output material.

RESULTS

Validation of Scale-Down Process

To ensure that the performance of the scale-down fraction IV fractionation process was representative of that used in production, several parameters of the scale-down process were compared with those of the manufacturing process. The ethanol concentration, pH, and temperature during scale-down process were 39.8±0.4 (% w/v), 6.0±0.1, and -5.5°C, respectively, which were all within the manufacturing specifications.

To ensure that the scale-down pasteurization procedure was equivalent to that used in the manufacture of 20% human albumin in a production facility, physicochemical analyses of samples before and after pasteurization were conducted. A comparison of the pH, protein, and content of albumin aggregate in the scale-down and production pasteurization process confirmed that the scale-down pasteurization run was representative of the manufacturing process (data not shown). The pH and protein content in the scale-down process were the same as those observed in production batches, and the content of the albumin aggregate also increased on pasteurization to levels routinely observed in production.

Inactivation of Enveloped Viruses (BHV and BVDV) during Fraction IV Fractionation Process

The partitioning profile of BHV and BVDV after fraction IV fractionation was assessed in order to evaluate the efficacy of the fraction IV fractionation process in eliminating these enveloped viruses. After the separation of the precipitates, the titers of the viruses in the resulting supernatant and the fraction IV paste were analyzed (Table 1). No infectious virus was detected in the supernatant, thereby indicating the complete removal of these viruses during fraction IV fractionation. Furthermore, no infectious virus was found to be present in the paste. These results indicate that all the spiked viruses were inactivated during fraction IV fractionation

Table 1. Reduction of BHV and BVDV during fraction IV fractionation.

Exp. No.	Sample	Total virus titer (Log_{10} $TCID_{50}$)	
		BHV	BVDV
1.	Virus spiked	8.4	7.9
	Starting material spiked with virus	8.3	7.9
	Fraction IV paste	ND*	ND
	Fraction IV supernatant	ND	ND
2.	Virus spiked	8.6	7.8
	Starting material spiked with virus	8.4	7.9
	Fraction IV paste	ND	ND
	Fraction IV supernatant	ND	ND

*No infectious virus was detected.

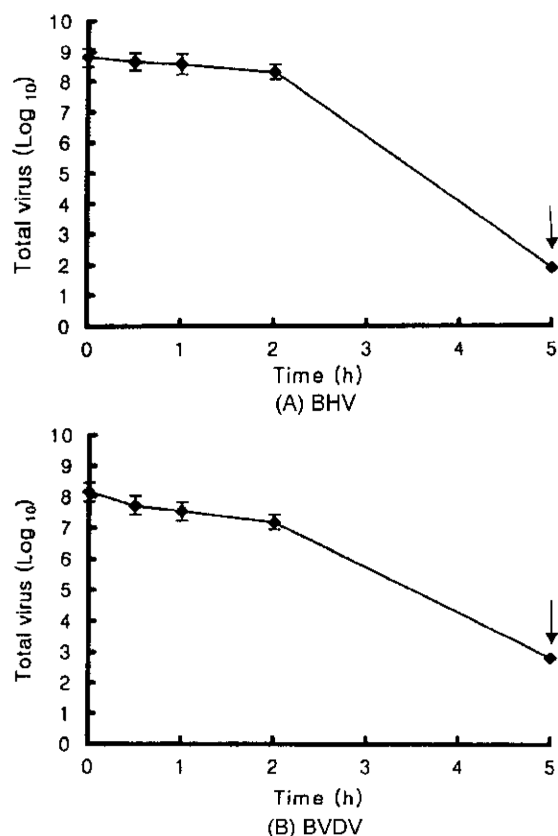


Fig. 2. Kinetics of inactivation of BHV and BVDV during fraction IV fractionation.
The arrows indicate the detection limits of the quantitative assay.

by the addition of ethanol, thus the mechanism of reduction was inactivation rather than partitioning under these experimental conditions. Thereafter, the inactivation kinetics of the enveloped viruses under the conditions of fraction IV fractionation (ethanol 40%, pH 6.0, -5.5°C) were studied (Fig. 2). BHV and BVDV showed a biphasic reduction. As such, they were insignificantly inactivated during the first 2 h of treatment, yet completely inactivated after 5 h of treatment. No residual infectivities of BHV and BVDV were found after 5 h of treatment. The log reduction factors achieved were ≥6.9 for BHV and ≥5.2 for BVDV.

Partitioning of Non-Enveloped Viruses (EMCV and PPV) during Fraction IV Fractionation Process

The partitioning profile of EMCV and PPV after fraction IV fractionation was assessed in order to evaluate the efficacy of the fraction IV fractionation process in eliminating non-enveloped viruses. EMCV and PPV were effectively removed during the fraction IV fractionation process (Table 2). After the separation of the precipitates, most of the viruses were recovered in the fraction IV paste, thereby indicating that the mechanism of reduction in the supernatant of fraction IV was partitioning rather than

Table 2. Partitioning of EMCV and PPV during fraction IV fractionation.

Exp. No.	Sample	Total virus titer ($Log_{10}TCID_{50}$)	
		EMCV	PPV
1.	Virus spiked	9.3	7.8
	Starting material spiked with virus	8.4	7.9
	Fraction IV paste	7.7	8.0
	Fraction IV supernatant	3.4	4.1
	Log reduction factor	5.0	3.8
2.	Virus spiked	9.3	7.8
	Starting material spiked with virus	8.9	8.0
	Fraction IV paste	7.5	7.7
	Fraction IV supernatant	4.1	3.9
	Log reduction factor	4.8	4.1

inactivation. The log reduction factors achieved were 4.9 for EMCV and 4.0 for PPV.

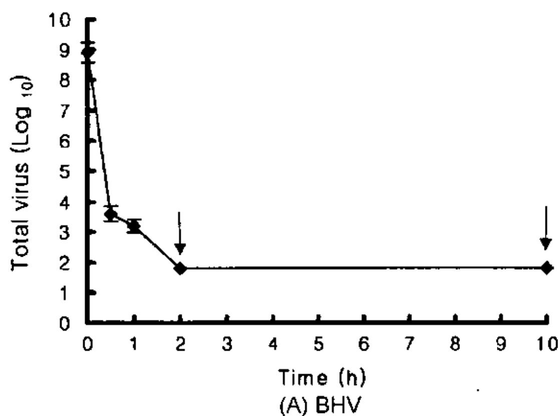
Virus Inactivation during Pasteurization

The effectiveness of a 60°C heat treatment for 10 h in inactivating viruses was determined. BHV was completely inactivated from an initial titer of 8.9 log_{10} $TCID_{50}$ to undetectable levels within 2 h of incubation (Fig. 3A). The log reduction factor obtained was ≥7.1. BVDV was completely inactivated from an initial titer of 8.2 log_{10} $TCID_{50}$ to undetectable levels within 1 h of treatment (Fig. 3B). The log reduction factor achieved was ≥6.4.

EMCV was completely inactivated from an initial titer of 9.8 log_{10} $TCID_{50}$ to undetectable levels within 5 h of incubation (Fig. 4A). The log reduction factor obtained was ≥6.8. However, PPV was incompletely inactivated with considerable residual infectivity remaining even after 10 h of treatment (Fig. 4B). The log reduction factor achieved was 1.7.

DISCUSSION

Human albumin solutions manufactured using cold ethanol fractionation and pasteurization procedures have an excellent viral safety record based on 50 years of clinical use [7, 17, 26]. This excellent safety record with respect to viral transmission by human albumin solutions is attributed to a combination of several factors: (i) careful selection of



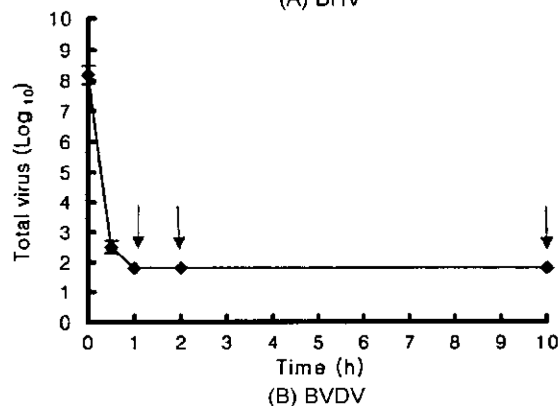
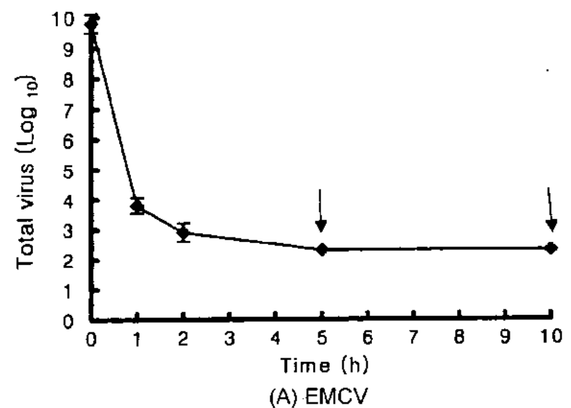


Fig. 3. Kinetics of inactivation of BHV and BVDV during pasteurization at 60°C for 10 h.
The arrows indicate the detection limits of the quantitative assay.



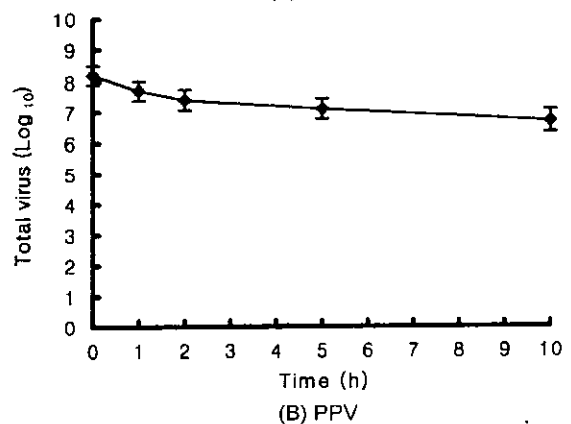


Fig. 4. Kinetics of inactivation of EMCV and PPV during pasteurization at 60°C for 10 h.
The arrows indicate the detection limits of the quantitative assay.

donors, (ii) careful screening of units donated for known infectious viruses, (iii) viral removal and inactivation by methods used during the manufacture of albumin, and (iv) testing of products and intermediates for viral markers. Although albumin solutions have proven to be safe, it is now essential that the potency of the various steps involved in the entire manufacturing process to remove and/or inactivate viral infectivity must be evaluated [12]. Accordingly, a validation study can provide evidence whether a production process can effectively inactivate and/or remove viruses which are either known to contaminate the starting materials, or which could conceivably do so, and whether the production process can inactivate and/ or remove a novel or unpredictable virus contamination. Therefore, this study was designed to evaluate the efficacy of the cold ethanol fractionation and pasteurization steps in the removal and/or inactivation of several blood-born model viruses differing in their sensitivity to physical and chemical agents, the nature of their genomes, and their size.

The most essential clue for a validation study is to establish the validity of the scale-down step. All the parameters obtained for the scale-down process during this study were within the manufacturing specifications, thereby indicating that all the scale-down processes were well designed to mimic as closely as possible the full manufacturing scale 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 in the fractionation is 40% and in some steps only 8-25%. In addition to the viricidal effect of ethanol, the partitioning of viruses during fractionation is also important in determining the effectiveness of this process in the production of safe plasma products [19]. The mechanism of reduction in eliminating enveloped viruses during fraction IV fractionation was found to be inactivation rather than partitioning. BHV and BVDV were completely inactivated from an initial titer of 8.6 $\log_{10}$ $TCID_{50}$ and 8.5 $\log_{10}$ $TCID_{50}$, respectively, to undetectable levels during incubation with 40% ethanol. The inactivation kinetics of BHV and BVDV showed a slow initial phase followed by a faster phase. These biphasic inactivation curves indicate that these viruses may have been present in an aggregated form, with outer particles protecting those inside the aggregate from the effect of ethanol. These viral aggregates may have been present in the virus stock or were possibly ethanol-induced as a result of lipid envelope denaturation. Another possible explanation for the biphasic inactivation curves is that the enveloped viruses may have inherent resistance to these experimental conditions (ethanol 40%, pH 6.0, -5.5°C) at least for 2 h.

The non-enveloped viruses were more resistant to ethanol than the enveloped viruses. PPV and EMCV were partitioned into the paste during fraction IV fractionation, thereby indicating that the mechanism of reduction for non-enveloped viruses during cold ethanol fractionation was partitioning. Most of the PPV was recovered in the precipitates, indicating that this virus is highly resistant to ethanol. However, less than 10% of the infectivity of the EMCV spiked into the starting material was recovered in the fraction IV paste, showing that the infectivity of EMCV is reduced by the addition of ethanol during the fraction IV fractionation process.

The principal method for the inactivation of virus contamination in plasma products is heating in a liquid for at least 10 h at 60°C. Heat treatment, which destroys the viral envelope, has been demonstrated by a number of investigators to effectively inactivate viral contaminants in the preparation of many plasma-derived products [9, 18, 22]. The inactivation kinetics of enveloped viruses such as BHV and BVDV showed that all the viruses were completely inactivated during the pasteurization process, with no residual viruses being detected on completion of the pasteurization process. The time needed for the complete inactivation of BHV and BVDV in these experimental conditions was 2 h and 1 h, respectively. Also EMCV was completely inactivated within 5 h of pasteurization under the same experimental conditions. From these results it can be concluded that pasteurization is a robust and effective step in eliminating enveloped viruses aw well as EMCV. These results are consistent with those of other investigators that show pasteurization to be an effective means of providing an extra measure of viral safety in plasma derivatives [9, 10, 21, 30]. Since the viral load was reduced to undetectable levels within 2 h of the total 10 h of incubation in these experiments, it can also be concluded that the extent of viral inactivation was independent of the initial viral load of BHV and BVDV.

Parvovirus is known to be very resistant to many physicochemical agents [25, 29]. The result presented in the present study indicated that PPV, a model virus for the human parvovirus B19, is highly resistant to heat treatment. This result is also consistent with those from previous studies [2, 6, 27].

The results of this study indicate that the fraction IV fractionation and pasteurization steps involved in manufacturing human albumin produce a significant removal and inactivation of several model viruses. The cumulative log reduction factors of both processes, ≥13.9 for BHV, ≥11.3 for BVDV, ≥11.2 for EMCV, and 5.7 for PPV, were several magnitudes greater than the potential virus load of current plasma pools. Accordingly these results indicate that the production process for human albumin has sufficient virus-reducing capacity to achieve a high margin of virus safety and that the excellent safety record of albumin can be explained by the effectiveness and robustness of these two processes.

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