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Improvement of Virus Safety of a Human Intravenous Immunoglobulin by Low pH Incubation

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Abstract In order to increase the virus safety of a human intravenous immunoglobulin (IVIg) that was manufactured by a successive process of cold ethanol fractionation, polyethylene glycol precipitation, and pasteurization (60 °C heat treatment for 10 h), a low pH incubation process (pH 3.9 at 25 °C for 14 days) was employed as the final step. The efficacy and mechanism of the fraction III cold ethanol fractionation, pasteurization, and low pH treatment steps in the removal and/or inactivation of blood-borne viruses were closely examined. A variety of experimental model viruses for human pathogenic viruses, including the Bovine herpes virus (BHV), Bovine viral diarrhoea virus (BVDV), Murine encephalomyocarditis virus (EMCV), and Porcine parvovirus (PPV), were selected for this study. The mechanism of reduction for the enveloped viruses (BHV and BVDV) during fraction III fractionation was both inactivation and partitioning, however, it was partitioning in the case of the nonenveloped viruses (EMCV and PPV). The log reduction factors achieved during fraction III fractionation were ≥6.7 for BHV, ≥4.7 for BVDV, 4.5 for EMCV, and 4.4 for PPV. Pasteurization was found to be a robust and effective step in inactivating all the viruses tested. The log reduction factors achieved during the pasteurization process were ≥7.5 for BHV, ≥4.8 for BVDV, 3.0 for EMCV, and 3.3 for PPV. A low pH incubation was very effective in inactivating the enveloped viruses as well as EMCV. The log reduction factors achieved during low pH incubation were ≥7.4 for BHV, ≥3.9 for BVDV, 5.2 for EMCV, and 2.0 for PPV. These results indicate that the low pH treatment successfully improved the viral safety of the final products.

Key words: Human intravenous immunoglobulin, low pH incubation, cold ethanol fractionation, pasteurization, virus removal and inactivation

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Human plasma, which is the liquid portion of blood, is made up of a complex mixture of proteins. A number of plasma components, including albumin, immunoglobulins, and coagulation factors, have been isolated for therapeutic use. In particular, intravenous immunoglobulin (IVIg) products have been used successfully for a number of indications, including primary and secondary immunodeficiencies, pediatric AIDS, infection in low birth weight infants, bone marrow transplantation, chronic lymphocytic leukemia, immune thrombocytopenic purpura, Kawasaki's syndrome, chronic inflammatory demyelinating polyneuropathies, Guillain-Barre syndrome, along with intractable seizure disorders [6, 31, 35].

The Cohn fractionation process is a well-established method for separating plasma into many protein components [3]. This separation method is often referred to as a 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. It has also been reported that partitioning as well as inactivation of viruses occur during the cold ethanol fractionation process [16, 22].

Although IVIg products have been regarded as generally safe against viruses, which is often attributed to the natural clearance effect during the cold ethanol fractionation and to the neutralizing effect by immunoglobulins [4, 39], a concern regarding the viral safety of these products has been raised by reports of transmission of hepatitis C virus and the development of chronic hepatitis C in patients treated with IVIg [18, 25, 36, 38]. These transmissions were associated with products manufactured by processes that differed in a number of aspects, but cold ethanol fractionation was included in all cases. This suggests that the cold ethanol fractionation process for immunoglobulin has a limited potential only to remove or inactivate HCV contaminants, when GMP (Good Manufacturing Practice)

failures are not included as a possible cause of such contamination [2]. For this reason, implementation of multiple viral removal and/or inactivation steps has been highly recommended for plasma-derivative manufacturing.

IVIg solutions for clinical use are manufactured from the fraction II paste by the cold ethanol fractionation of large quantities of pooled plasma from Greencross PD Co. This IVIg process contains a pasteurization step to inactivate these viruses. Recently, an additional low pH treatment of final products has been incorporated into the IVIg process to further enhance the viral safety. An 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 which have high potential for viral contamination from the source material [12, 17]. Accordingly, this study was designed to evaluate the efficacy and mechanism of the fraction III cold ethanol fractionation, pasteurization, and low pH incubation steps, employed in the manufacture of IVIg from human plasma, in the removal and/or inactivation of several model viruses.

MATERIALS AND METHODS

Plasma and IVIG Manufacturing Process

Samples for virus inactivation and partitioning studies were prepared from a source plasma that was derived 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 the polymerized chain reaction. IVIg was prepared by a process of cold ethanol fractionation according to the Cohn-Oncley procedures [3, 27]. As shown in Fig. 1, after thawing the frozen human plasma, the insoluble protein precipitate (Cryopaste) was separated, and the cryo-poor plasma protein components were then fractionated using cold ethanol. Fraction I+II+III paste was washed in order to remove the residual proteins remaining in the mother liquor of the paste. The washed fraction of I+II+III, designated as fraction I+II+IIIw, was further fractionated. The dissolved fraction II for immunoglobulins was ultrafiltered to remove the residual ethanol. The resulting bulk solution was stabilized with 33% sorbitol and pasteurized at 60°C for 10 h for virus inactivation. After dialysis of the pasteurized solution to remove the stabilizer, the impurities containing polymers were precipitated by adding polyethylene glycol (PEG 4000) to as much as 4%. After removing the precipitate from the solution, PEG 4000 was added to the filtered solution up to 12% to precipitate immunoglobulins. The precipitate was collected by centrifugation and then dissolved in water for injection. After adding maltose to 10% as a stabilizer, the pH of the final solution was adjusted to 3.9. After filling into bottles, the IVIg was incubated at 25°C for 14 days.

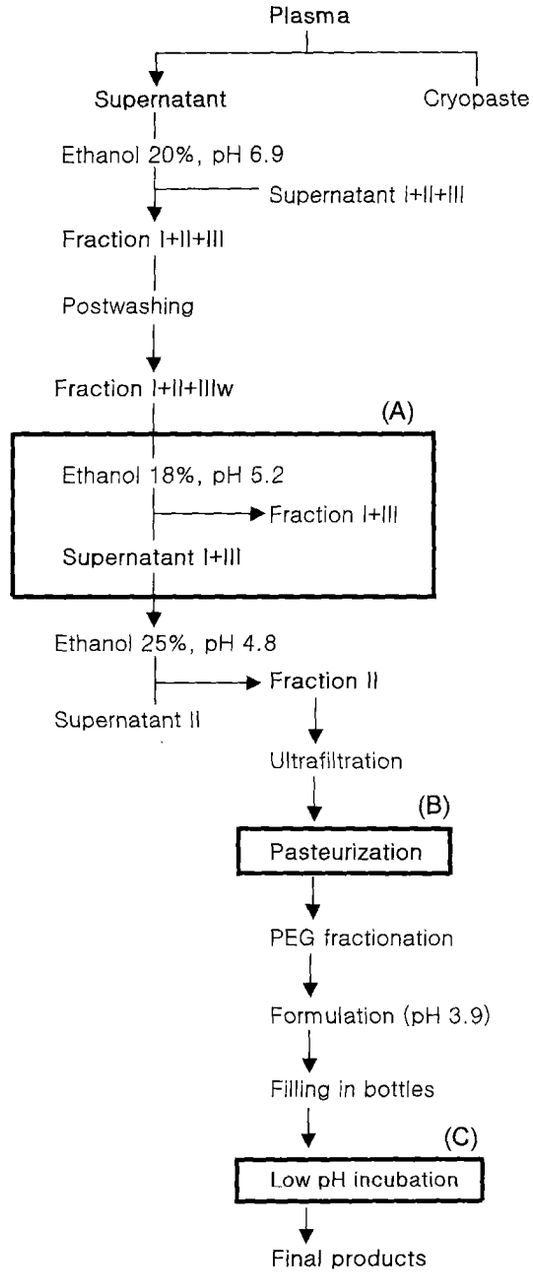


Fig. 1. Flow diagram of the plasma fractionation and manufacturing process for IVIg.
The boxes indicate the validation steps evaluated for virus removal/inactivation.

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 to display a significant resistance to physical or chemical agents [12, 16]. The viruses selected were as follows.

Bovine herpes virus (BHV) - enveloped, large, double-stranded DNA virus (ATCC VR-188) [19]. Herpesviruses can remain as latent infections within the 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 a representative of this class of virus that could be obtained in a sufficient titer for validation studies, and it was 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 Dulbecco's modified Eagle's medium (HG DMEM) containing 2% FBS plus nonessential amino acids (NEAA).

Bovine viral diarrhoea virus (BVDV) - enveloped, medium-sized, single-stranded RNA virus (ATCC VR-534) with a medium resistance to physicochemical reagents [19]. BVDV belongs to the Flaviviridae family, which also includes the hepatitis C virus [9, 28]. 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 2% FBS plus NEAA.

Murine encephalomyocarditis virus (EMCV) - nonenveloped, small, single-stranded RNA virus (ATCC VR-129B) with a medium to high resistance to physicochemical reagents [19]. EMCV provides a severe test to validate the process for removal and/or inactivation of viruses. EMCV belongs to the Picornaviridae family, which also includes the hepatitis A virus [15]. For the propagation and titration of EMCV, Vero C1008 cells (ATCC CRL-1586) were used, which were grown in HG DMEM containing 2% FBS plus NEAA.

Porcine parvovirus (PPV) - nonenveloped, small, single-stranded DNA virus (ATCC VR-742) [19]. This virus has a high resistance to various physicochemical reagents and it is a known contaminant of porcine blood. Therefore, it provides a severe test for validation of a process as for 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 2% 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 with at least eight 0.25 ml replicates of the appropriate dilution of each sample or the positive control. Negative control wells were mock-infected with 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 1 ml 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 III fractionation, pasteurization, and low pH treatment 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 precisely where spiking the virus into the starting material resulted in a loss in the virus titer.

Virus Partitioning by Fraction III Fractionation

A 96-ml aliquot of the fraction I+II+IIIw suspension was spiked with 9 ml of the virus. An 8-ml aliquot of the sample was then immediately removed to determine the viral titer in the spiked starting material. The remaining material was continuously supplemented with 95% ethanol at −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, Germany). The resulting supernatant was collected, and the paste I+III fraction was resuspended in the tissue culture medium with the same volume as that of the supernatant. An aliquot from each sample was immediately titrated.

Virus Inactivation during Pasteurization

The dissolved fraction II solution was dialyzed against water to remove the residual ethanol, and 35 ml of the aliquot solution was then spiked with 3.5 ml of the virus. Sorbitol was added to the virus-spiked solution by as much as 33% (w/v). After adjusting the pH to 5.0, the resulting solution was pasteurized at 60±0.5°C for 10 h. Samples were removed at different times and then immediately titrated.

Virus Inactivation during Low pH Incubation

A 54-ml aliquot of the final IVIg solution was spiked with 6 ml of the virus and the pH of the solution was then adjusted to 3.9. Ten ml of the aliquot sample was then immediately removed to determine the viral titer in the spiked starting material. The remaining solution was incubated at 25°C for 14 days. Samples were removed at various times and then 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, 17]. 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, and aII=the titer of the virus in the output material.

Stability Test of IVIG

The influence of low pH incubation on the stability of IVIg was studied by measuring antibody potency against measles and hepatitis B viruses. Measles virus antibody content was measured by a plaque reduction neutralization method with the anti-measles serum (NIBSC, U.K.). The challenged virus was the Schwarz strain (Chiron, U.S.A.) and the cell used was the Vero cell (ATCC CCL81). For the titration of hepatitis B virus antibody, the ELISA method was applied using an ES-300 instrument and hepatitis B virus antibody (Boehringer Mannheim, Germany).

Some aggregates, particularly those generated by heating at neutral pH, possess complement binding activity and fix complement by a mechanism similar to antigen-antibody complexes. The presence of this type of aggregated immunoglobulins is known to cause an adverse reaction [30]. Therefore, the anticomplementary activity was measured before and after the low pH incubation. The anticomplementary activity assay is based on a complement-mediated red cell lysis. The hemolytic unit of complement activity (CH_{50}) is the amount of complement that will produce the lysis of 2.5×10^{8} out of a total of 5×10^{8} optimally sensitized red blood cells in the given reaction conditions. In this test, one volume of two CH_{50} units of guinea pig complement (Denka Seiken, Japan) was added to one-half sequentially diluted test samples and then incubated at 37°C for 2 h. After incubation, an equal volume of complement solution of sensitized sheep red blood cell was added and then incubated for 30 min. Inhibition of hemolysis was measured by a spectrophotometer at 541 nm. The anticomplementary activity value was expressed on the basis of consumption rate of complement (CH_{50}) unit per 50 mg IgG protein.

Polymer contents of IVIg before and after the low pH incubation were determined by high performance liquid chromatography (HPLC) (Waters, Millenium 32) using a TSK gel G3000 column (7.5 cm×60 cm, Tosoh, Japan). Phosphate buffered saline (40 mM, pH 6.8) was used as the mobile phase, and the flow rate was 0.6 ml/min.

RESULTS

Validation of the Scale-Down Process

To ensure that the performance of the scale-down fraction III fractionation process was representative of those 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 the scale-down process were 18±0.3 (% w/v), 5.2±0.1, and −5.5°C, respectively, and they were within the manufacturing specifications.

To assure that the scale-down pasteurization procedure was equivalent to that used in manufacturing IVIg in the production facility, physicochemical analyses of samples were conducted before and after pasteurization. A comparison of the pH, protein, and IVIg aggregate content in the scale-down and production pasteurization processes confirmed that the scale-down pasteurization run was a 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 IVIg aggregate on pasteurization also increased to levels routinely observed in production.

Virus Reduction during the Fraction III Fractionation Process

The partitioning profile of BHV, BVDV, EMCV, and PPV after fraction III fractionation was assessed in order to evaluate the efficacy of the fraction III fractionation process

Table 1. Reduction of viruses during fraction III fractionation.

Sample	Total virus titer ($\log_{10}$ $TCID_{50}$)			
	BHV	BVDV	EMCV	PPV
Virus-spiked	9.7±0.2	6.5±0.3	9.0±0.3	8.1±0.2
Starting material spiked with virus	9.5±0.3	6.6±0.2	9.1±0.1	7.7±0.4
Fraction III paste	5.8±0.1	5.3±0.1	8.7±0.2	7.7±0.3
Fraction III supernatant	ND[a] (=2.8)[b]	ND (=1.9)	4.6±0.2	3.3±0.1
Reduction factor ($\log_{10}$)	≥6.7	≥4.7	4.5	4.4

[a]No infectious virus was detected.

[b]These values were calculated using a theoretical minimal detection level of infectious virus with a 95% confidence level.

in eliminating viruses. All the viruses tested were effectively removed during the fraction III fractionation process (Table 1). No infectious BHV or BVDV was detected in the supernatant, indicating a complete removal of these enveloped viruses during the fraction III fractionation process. The log reduction factors achieved were ≥6.7 for BHV and ≥4.7 for BVDV.

The nonenveloped viruses (EMCV and PPV) were also effectively removed during the fraction III fractionation process (Table 1). After the separation of the precipitates, most of the viruses were recovered in the fraction III paste. The log reduction factors achieved were 4.5 for EMCV and 4.4 for PPV.

Virus Inactivation during Pasteurization

The effectiveness of 60°C heat treatment for 10 h to inactivate viruses was determined. BHV was completely inactivated from an initial titer of 9.5 $\log_{10}$ $TCID_{50}$ to undetectable levels within 2 h of incubation (Fig. 2A), and the log reduction factor obtained was ≥7.5. BVDV was completely inactivated from an initial titer of 6.8 $\log_{10}$ $TCID_{50}$ to undetectable levels within 2 h of treatment (Fig. 2B), and the log reduction factor achieved was ≥4.8.

EMCV was inactivated from an initial titer of 8.7 $\log_{10}$ $TCID_{50}$ to 5.7 $\log_{10}$ $TCID_{50}$ with residual infectivity remaining even after 10 h of treatment (Fig. 3A), and the log reduction factor obtained was 3.0. PPV was also incompletely inactivated from an initial titer of 7.9 $\log_{10}$ $TCID_{50}$ to 4.6 $\log_{10}$ $TCID_{50}$ after 10 h of incubation (Fig. 3B), and the log reduction factor achieved was 3.3.

Virus Inactivation during Low pH Incubation

The effectiveness of low pH incubation at pH 3.9 and 25°C for 14 days in inactivating viruses was determined. BHV was rapidly inactivated from an initial titer of 8.6 $\log_{10}$ $TCID_{50}$ to 2.6 $\log_{10}$ $TCID_{50}$ within 1 h of incubation and then completely inactivated to undetectable levels within 5 days of incubation (Fig. 4A). The log reduction factor obtained was ≥7.4. BVDV was completely inactivated from an initial titer of 5.8 $\log_{10}$ $TCID_{50}$ to undetectable levels within 9 days of incubation (Fig. 4B), and the log reduction factor achieved was ≥3.9.

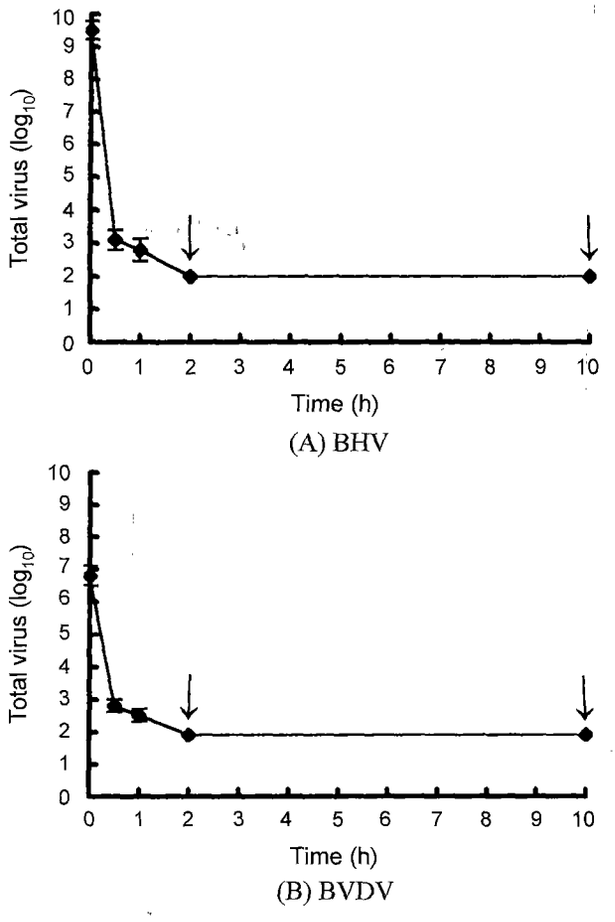


Fig. 2. Kinetics of inactivation of BHV and BVDV during pasteurization at 60°C for 10 h.
The arrows indicate the detection limits of the quantitative assay.

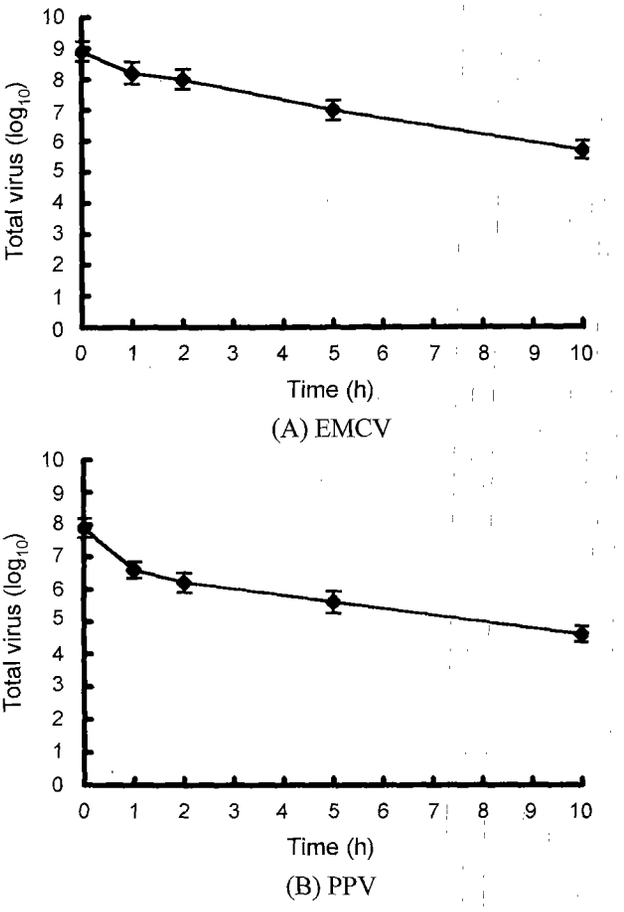
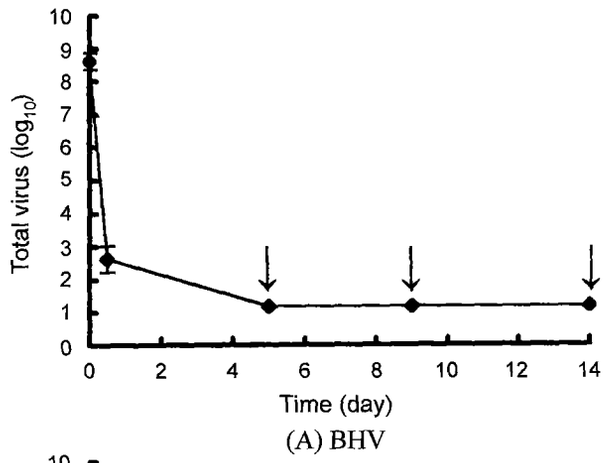


Fig. 3. Kinetics of inactivation of EMCV and PPV during pasteurization at 60°C for 10 h.



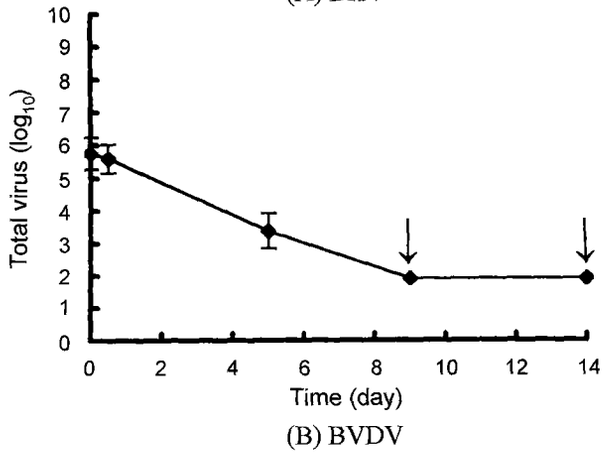
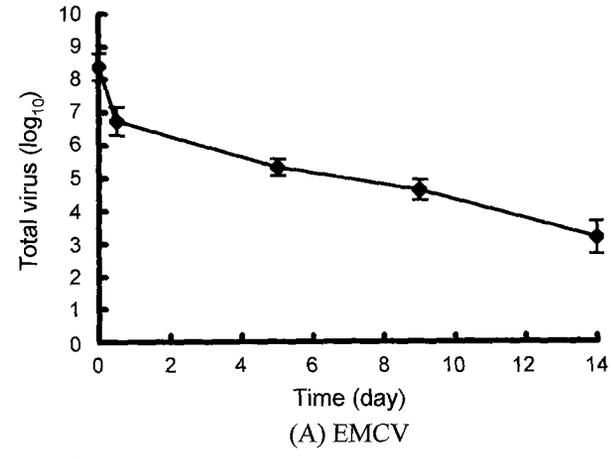


Fig. 4. Kinetics of inactivation of BHV and BVDV during incubation at pH 3.9 and 25°C for 14 days.
The arrows indicate the detection limits of the quantitative assay.



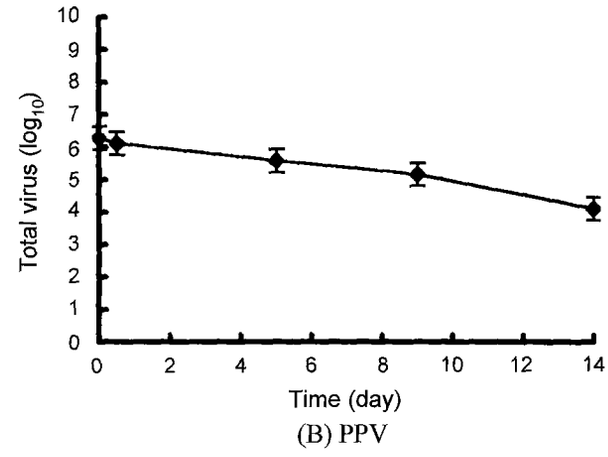


Fig. 5. Kinetics of inactivation of EMCV and PPV during incubation at pH 3.9 and 25°C for 14 days.

EMCV was inactivated from an initial titer of 8.4 log_{10} $TCID_{50}$ to 3.2 log_{10} $TCID_{50}$ with slightly residual infectivity remaining after 14 days of incubation (Fig. 5A), and the log reduction factor obtained was 5.2. PPV was also incompletely inactivated from an initial titer of 6.3 log_{10} $TCID_{50}$ to 4.3 log_{10} $TCID_{50}$ after 14 days of incubation (Fig. 5B), and the log reduction factor achieved was 2.0.

Stability of IVIg After Low pH Treatment

The influence of low pH incubation on the stability of IVIg was studied (Table 2). Antibody titers against measles and hepatitis B viruses were not changed during low pH incubation at 25°C for 14 days. To further assess IVIg stability, IVIg solutions after low pH incubation were stored at 4°C for 24 months and the potency of the measles antibody was periodically measured. The titers of measles antibody were maintained up to 24 months.

Table 2. Effect of low pH incubation on the stability of IVIg.

Items	Low pH incubation at 25°C for 14 days		Long-term stability after low pH treatment[a]			
	Before	After	6 months	12 months	18 months	24 months
Antibody titer						
Anti-measles (U/150 mg)	18.4	17.4	20.0	17.3	22.2	18.6
Anti-hepatitis B (U/g)	19.0	18.9	ND[b]	ND	ND	ND
Anticomplementary activity (CH_{50} U/50 mg)	13.3	13.9	13.8	13.9	14.1	13.8
Polymer (%)	0.10	0.10	0.07	0.10	0.13	0.07

[a]IVIg solutions after low pH incubation were stored at 4°C for 24 months and the stability of IVIg was periodically measured.
[b]Not determined.

Changes of anticomplementary activity in IVIg were investigated using a complement-mediated assay of red cell lysis, and was found not changed during low pH incubation and subsequent storage at 4°C up to 24 months.

To further assess protein stability, the polymer content of IVIg was analyzed by size-exclusion HPLC and was found to be unchanged during low pH incubation and subsequent storage at 4°C up to 24 months.

DISCUSSION

Many steps are taken in the preparation of IVIg and other plasma-derived products to ensure their safety from viral contamination, but the most important steps are those phases of the manufacturing process in which viral contaminants are either inactivated or separated from the final products. The cold ethanol fractionation process is a well-established method for separating plasma into as many protein components as possible [3, 27] and partitioning viral contaminants from the final products [22]. In addition to the partitioning effect of viruses, the viricidal effect exerted by the added ethanol in the fractionation is also important in determining the effectiveness of this process in the production of safe plasma products [16]. The mechanism of reduction in eliminating enveloped viruses during fraction III fractionation was found to be both partitioning and inactivation. Although no infectious viruses were detected in the supernatant, the recovery of BHV or BVDV in the fraction III paste was significantly low. The differences of total virus titer before and after the fraction III fractionation were about 3.7 $\log_{10}$ $TCID_{50}$ for BHV and 1.3 $\log_{10}$ $TCID_{50}$ for BVDV, which showed that infectivities of these viruses were also reduced by the added ethanol during the fraction III fractionation process. The nonenveloped viruses were more resistant to ethanol than the enveloped viruses. PPV and EMCV were effectively partitioned into the paste during fraction III fractionation; furthermore, most of these viruses were recovered in the precipitates, thus indicating that the mechanism of reduction for nonenveloped viruses during fraction III fractionation was that of partitioning. Not all cold ethanol fractionation steps were tested in this study, therefore, the actual virus reduction might be larger than that demonstrated in this study.

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 [10, 21, 24]. In the present study, 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 detected on completion of the pasteurization process. The time needed for complete inactivation of BHV and BVDV in these experimental conditions was 2 h. These results led us to conclude that the pasteurization process was a robust and effective step in eliminating enveloped viruses. These results are consistent with those of other investigators who showed pasteurization to be an effective means to provide an extra measure of viral safety in plasma derivatives [10, 11, 23, 37]. Since the viral load was reduced to undetectable levels within 2 h of the total 10 h incubation time period in these experiments, it could also be concluded that the extent of viral inactivation was independent of the initial viral load of BHV and BVDV.

The results presented in the present study indicated that EMCV and PPV were highly resistant to heat treatment in this IVIg manufacturing condition. It is of interest to point out that residual infectivity of EMCV, a model virus for HAV, still remained even after 10 h of treatment during the manufacturing process of IVIg, although it was completely inactivated within 5 h of pasteurization during the process of manufacturing 20% albumin [16]. This difference was likely caused by the stabilizer, sorbitol, added for IVIg, which might stabilize EMCV as well.

Parvovirus is known to be very resistant to many physicochemical agents [29, 34]. The results presented in the present study indicated that PPV, a model virus for the human parvovirus B19, was highly resistant to heat treatment. This result is also consistent with those from previous studies [1, 5, 33].

Treatment of intramuscular immunoglobulin products (IMIG) at pH 4.0 in the presence of a low concentration of pepsin was originally developed to reduce both anticomplementary activity and contaminating enzymes of the contact activation system, in order to render these products to be suitable for intravenous administration [7]. Subsequently, it was shown that such treatment may also improve viral safety of IVIg [32]. Due to occasional reports made of HCV transmission by these products [18, 25, 36, 38], studies on the efficacy of low pH incubation in the inactivation of viruses from IVIg have mainly focused on the safety from HCV of IVIg that was prepared in the presence of pepsin [8, 14, 20, 26]. BVDV has been used as a surrogate for HCV in those studies requiring virus infectivity titration of numerous samples, thereby making it impractical in using HCV, which can only be tested in chimpanzees and marmosets. A previous report has shown that the lower the pH level and the higher the temperature, the greater the inactivation of viruses was achieved [20].

Green Cross PD Co. is currently manufacturing IVIg using PEG fractionation to reduce impurities instead of the pepsin treatment. In this study, a low pH incubation was performed in the absence of pepsin. The results of the stability test after low pH incubation at 25°C for 14 days and subsequent storage at 4°C for 24 months showed that IVIg was stable in solution at pH 3.9.

Table 3. Virus log reduction factors achieved during the manufacturing process of IVIg.

Process step	Lipid enveloped virus		Nonenveloped virus	
	BHV	BVDV	EMCV	PPV
Fraction III fractionation	≥6.7	≥4.7	4.5	4.4
Pasteurization	≥7.5	≥4.8	3.0	3.3
Low pH treatment	≥7.4	≥3.9	5.2	2.0
Cumulative log reduction factor	≥21.6	≥13.4	12.7	9.7

The inactivation kinetics of enveloped viruses during low pH incubation showed that all the viruses were completely inactivated during the incubation process, with no residual viruses being detected. The time needed for the complete inactivation of BHV and BVDV in these experimental conditions (pH 3.9, 25°C) was 5 days and 9 days, respectively. From these results, it can be concluded that incubation at pH 3.9 is a robust and effective step in eliminating enveloped viruses. The infectivity of nonenveloped viruses is much reduced at low pH treatment, with residual infectivity remaining after 14 days of incubation. Although most of EMCV was inactivated during the low pH incubation period, PPV was incompletely inactivated from an initial titer of 6.3 $\log_{10}$ $TCID_{50}$ to 4.3 $\log_{10}$ $TCID_{50}$, even after 14 days of treatment.

The cumulative virus reduction factor for the manufacturing process is determined from the sum of the individual virus reduction factors based on individual process steps of different physicochemical methods [12]. The cumulative virus reduction factors achieved for different viruses by the process steps which were evaluated are presented in Table 3. The results of this study indicate that the fraction III fractionation, pasteurization, and additional low pH incubation steps involved in manufacturing IVIg, produce a significant removal and inactivation of several model viruses. The cumulative log reduction factors of these processes were several magnitudes greater than the potential virus load of current plasma pools. Accordingly, these results indicate that the production process for IVIg with the additional treatment step at low pH has a sufficient virus-reducing capacity to achieve a high margin of virus safety.

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