Cold Ethanol Fractionation and Heat Inactivation of Hepatitis A Virus 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 fraction IV cold ethanol fractionation and pasteurization (60°C heat treatment for 10 h), involved in the manufacture of albumin from human plasma, in the removal and/or inactivation of the hepatitis A virus (HAV). Samples from the relevant stages of the production process were spiked with HAV and the amount of virus in each fraction then quantified using a 50% tissue culture infectious dose ($TCID_{50}$). HAV was effectively partitioned from albumin during the fraction IV cold ethanol fractionation with a log reduction factor of 3.43. Pasteurization was also found to be a robust and effective step in inactivating HAV, where the titers were reduced from an initial titer of 7.60 log $TCID_{50}$ to undetectable levels within 5 h of treatment. The log reduction factor achieved during pasteurization was ≥4.76. Therefore, the current results indicate that the production process for albumin has sufficient HAV reducing capacity to achieve a high margin of virus safety.

Keywords: human albumin, cold ethanol fractionation, pasteurization, HAV removal and inactivation

Human serum albumin has been used for clinical purposes for a long time. Yet, since the albumin is manufactured from human plasma, special precautions must be taken during the production of this protein to guard against the possibility of the product transmitting infectious diseases to recipients. The major risk associated with the use of blood products is viral infection, such as the Human immunodeficiency virus (HIV), Hepatitis A, B, C, and G viruses, and Human T-cell lymphotropic viruses (HTLV) I and II [1-3].

Albumin solutions for clinical use are usually manufactured based on the cold ethanol fractionation of large quantities of pooled plasma [4,5]. For example, the Green Cross Plasma Derivatives Company currently produces human albumin solutions according to the cold ethanol fractionation method (Fig. 1). After thawing frozen human plasma, the insoluble protein precipitate (Cryoprecipitate) is harvested and used as the raw material for manufacturing antihemophilic Factor Ⅷ. The plasma protein components are then fractionated by a stepwise increase of the ethanol concentration plus a decrease in the pH. The dissolved fraction V is concentrated by ultrafiltration, then the resulting bulk solution is heated at 60°C for 10 h to remove any unwanted proteins

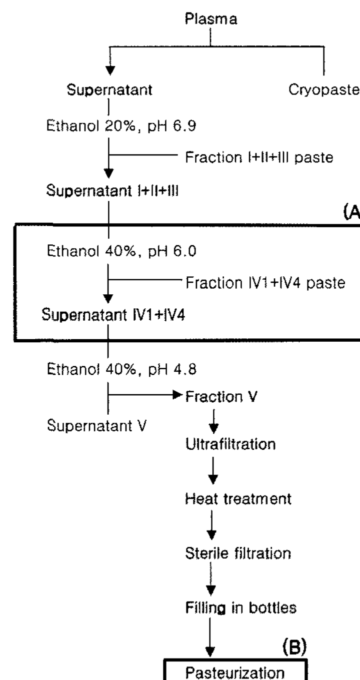

Fig. 1. Flow diagram of plasma fractionation and manufacturing process for albumin. The boxes indicate the validation steps evaluated for HAV removal/inactivation; (A) fraction IV fractionation, (B) pasteurization.

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Table 1. Partitioning of HAV during fraction IV fractionation

Sample	Total virus titer ($Log_{10} TCID_{50}$)	Log reduction factor
HAV spiked	8.13±0.15	-
Starting material spiked with HAV	7.90±0.16	-
Fraction IV paste	7.78±0.26	-
Fraction IV supernatant	4.70±0.16	3.43

These results are the mean values of three independent experiments.

and sterile-filtered using a 0.2 μm filter. The final solution is placed in bottles and then heated at 60±0.5°C for 10 h to inactivate any contaminating viruses.

Human albumin solutions manufactured using cold ethanol fractionation and pasteurization procedures have an excellent viral safety record based on 50 years of clinical use [6-8]. 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 blood 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. However, despite the proven safety record of this plasma product, the potency of the various steps involved in the manufacturing process, designed to remove and/or inactivate viral infectivity, still need to be evaluated as an essential and important component in establishing the safety of the biological products, especially when there is a high potential for the source material to be contaminated with a virus known to be pathogenic to humans [9,10]. Accordingly, several validation studies have already been conducted to evaluate the efficacy and mechanism of the process steps aimed at removing and/or inactivating any viruses. In such studies, several model viruses, such as the bovine viral diarrhea virus (BVDV; a model virus for the hepatitis C or hepatitis G virus), bovine herpes virus (BHV; a model virus for the human herpes virus, such as HHV-6, HHV-7, HHV-8, Epstein Barr virus, or HSV-1), murine encephalomyocarditis virus (EMCV; a model virus for the hepatitis A virus), porcine parvovirus (PPV; a model virus for human parvovirus), and human immunodeficiency virus (HIV-1), were chosen as examples of potential viral contaminants [5,11-15]. However, little has been studied about the hepatitis A virus (HAV). Therefore, the current study was designed to evaluate the efficacy and mechanism of cold ethanol fractionation and pasteurization in the removal and/or inactivation of HAV.

HAV is a member of the *Picornaviridae* family, which are non-enveloped, small (25-30nm), single-stranded RNA viruses with a medium to high resistance to physico-chemical inactivation [16]. Over the last decade, concern has emerged over HAV, stimulated by the reported transmission of this virus by clotting factor concentrates prepared from large plasma pools [17-19].

HAV strain HM/175/18f clone B (ATCC VR-1402) was prepared using FRhK-4 (ATCC CRL-1688) cells, as described in a previous report [20]. The HAV was titrated in 7-fold serial dilutions, ending in a quantal 50% tissue culture infectious dose ($TCID_{50}$) assay using FRhK-4 cells. As a part of the virus validation protocol, cytotoxicity, interference, and load titer tests were also performed, as described in previous report [20]. The virus reduction factor for each removal or inactivation step was defined as the $\log_{10}$ of the ratio of the virus load in the spiked starting material divided by the virus load in the post process material.

To evaluate the efficacy and mechanism of the fraction IV cold ethanol fractionation process in eliminating HAV, a virus spiking study was conducted using the scale-down process [5]. 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 hours, 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 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 on collection where required and an aliquot from each sample immediately titrated. (Table 1). HAV was effectively removed during the fraction IV fractionation process. However, after the separation of the precipitates, most of the viruses were recovered in the fraction VI paste, indicating that the mechanism of reduction in the supernatant of fraction IV was partitioning rather than inactivation. The log reduction factor achieved was 3.43. Ethanol is known to be both bactericidal and viricidal and is the principal precipitation agent in the cold ethanol fractionation used in the manufacture of plasma products. The highest concentration of ethanol in the fractionation is 40%. 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 [5,12]. It was found that the mechanism of reduction in eliminating enveloped viruses (such as HIV, BHV, and BVDV) during fraction IV fractionation was inactivation rather than partitioning. However, the mechanism of reduction for non-enveloped viruses (such as EMCV and PPV) was partitioning.

One of the principal methods for the inactivation of vi-

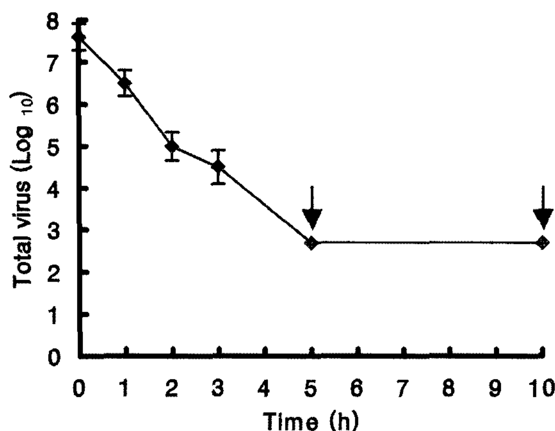

Fig. 2. Inactivation of HAV during pasteurization of albumin at 59±0.5°C for 10 h. The arrows indicate the detection limits of the quantitative assay.

rus 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 already been demonstrated to effectively inactivate viral contaminants in the preparation of many plasma-derived products [5,11,21,22]. As the worst-case conditions of pasteurization for the validation of virus inactivation, 59±0.5°C and 10 h were selected as the process temperature and process time, respectively. 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 the HAV solution, then a 6 mL load sample was removed for titration. The remaining material was heated in a water bath and equilibrated to 59±0.5°C. Samples were removed at different times over 10 h and an aliquot of each sample immediately titrated. The virus inactivation was kinetically evaluated as a function of the process time (Fig. 2). As such, the HAV was progressively inactivated from an initial titer of 7.60 $\log_{10}$ $TCID_{50}$ to undetectable levels within 5 h of incubation. The log reduction factor obtained was ≥4.76. Since the pasteurization experiment was conducted under worst-case conditions, it was concluded that pasteurization was a robust and effective measure for inactivating HAV. From a previous report using EMCV as the model virus for HAV, it was found that EMCV was also completely inactivated within 5 h of pasteurization [5]. Furthermore, the inactivation kinetics of enveloped viruses, such as BHV, BVDV, and HIV, revealed that all the viruses were completely inactivated with no residual viruses being detected on completion of the pasteurization process. The time needed for the complete inactivation of BHV, BVDV, and HIV was 2 h, 1 h, and 1 h, respectively [5,11]. However, PPV was incompletely inactivated with considerable residual infectivity remaining even after 10 h of treatment [5].

Human albumin solutions have an excellent viral safety record. For example, there has been no case of the transmission of HAV through albumin during more than 50 years of widespread clinical use, even in the years before HAV-positive blood could be eliminated by nucleic acid testing using a nested polymerized chain reaction [6-8]. The current study found that the fraction IV fractionation and pasteurization steps involved in manufacturing human albumin significantly removed and inactivated any HAV. Accordingly, these results indicate that the production process for human albumin has sufficient HAV-reducing capacity to achieve a high margin of virus safety and that the excellent safety record of albumin can indeed be explained by the effectiveness and robustness of this production process.

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REFRENCES

[1] Berkman S. A. (1988) Infectious complications of blood transfusion. *Blood Rev.* 2: 206-210.

[2] Schilt, U. (1989) Overview of viruses relevant to blood transfusion. *Curr. Stud. Hematol. Blood Transfus.* 56: 1-8.

[3] Cuthbertson, B., K. G. Reid, and P. R. Foster (1991) Viral contamination of human plasma and procedures for preventing virus transmission by plasma products. pp. 385-435. In: J. R. Harris (ed.). *Blood separation and plasma fractionation*. Willey-Liss., New York, USA.

[4] Cohn, E. J., L. E. Strong, W. L. Hughes Jr, D. J. Mulford, J. N. Ashworth, M. Melin, and H. L. Taylor (1946) Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of the proteins and lipoprotein components of biological tissues and fluids. *J. Am. Chem. Soc.* 68: 459-475.

[5] Kim, I. S., H. G. Eo, C. E. Chang, and S. Lee (2000) Partitioning and inactivation of viruses by cold ethanol fractionation and pasteurization during manufacture of albumin from human plasma. *J. Microbiol. Biotechnol.* 10: 858-864.

[6] Erstad, B. L. (1996) Viral infectivity of albumin and plasma protein fraction. *Pharmacotherapy* 16: 996-1001.

[7] Roberts, P. (1996) Virus safety of plasma products. *Rev. Med. Virol.* 6: 25-38.

[8] Tabor, E. (1999) The epidemiology of virus transmission by plasma derivatives: Clinical studies verifying the lack of transmission of hepatitis B and C viruses and HIV type 1. *Transfusion* 39: 1160-1168.

[9] Federal Health Office and Paul Ehrlich Institute Federal Office for Sera and Vaccines (1994) Notice on the registration of drugs: Requirements for validation studies to demonstrate the virus safety of drugs derived from human blood or plasma. *Bundesanzeiger* 84: 4742-4744.

[10] International Conference on Harmonisation (1998) Guidance on viral safety evaluation of biotechnology products

derived from cell lines of human or animal origin; Availability. *Federal Resister* 63(185): 51074-51084.
[11] Kim, I. S., H. G. Eo, C. W. Park, C. E. Chang, and S. Lee (2001) Removal and inactivation of human immunodeficiency virus (HIV-1) by cold ethanol fractionation and pasteurization during the manufacture of albumin and immunoglobulins from human plasma. *Biotechnol. Bioprocess Eng.* 6: 25-30.
[12] Morgenthaler J. J. and A. Omar (1993) Partitioning and inactivation of viruses during isolation of albumin and immunoglobulins by cold ethanol fractionation. *Dev. Biol. Stand.* 81: 185-90.
[13] Henin Y, V. Marechal, F. Barre-Sinoussi, J. C. Chermann, and J. J. Morgenthaler (1988) Inactivation and partition of human immunodeficiency virus during Kistler and Nitschmann fractionation of human blood plasma. *Vox Sang.* 54: 78-83.
[14]Wells M. A., A. E. Wittek, J. S. Epstein, C. Marcus-Sekura, S. Daniel, D. L. Tankersley, M. S. Preston, and G. V. Quinnan Jr (1986) Inactivation and partition of human T-cell lymphotrophic virus, type III, during ethanol fractionation of plasma. *Transfusion* 26: 210-213.
[15] Yei, S., M. W. Yu, and D. L. Tankersley (1992) Partitioning of hepatitis C virus during Cohn-Oncley fractionation of plasma. *Transfusion* 32: 824-828.
[16] Andrewes, C. (1989) *Andrewes' Viruses of Vertebrates*. pp. 120-145. Balliere Tindal, London, UK.
[17] Johnson, Z., L. Thornton, A. Tobin, E. Lawlor, J. Power, I. Hillary, and I. Temperley (1995) An outbreak of hepatitis A among Irish haemophiliacs. *Int. J. Epidermol.* 24: 821-828.
[18] Robinson, S. M., H. Schwinn, and A. Smith (1992) Clotting factors and hepatitis A. *Lancet* 340: 1465.
[19] Temperley, I. J., K. P. Cotter, T. J. Walsh, J. Power, and I. B. Hillary (1992) Clotting factors and hepatitis A. *Lancet* 340: 1466.
[20] Kim, I. S., Y. W. Choi, S. R. Lee, Y. Kang, K. M. Lee, D. H. Park, H. S. Woo, and S. Lee (2002) Removal and inactivation of hepatitis A virus during manufacture of urokinase from human urine. *Biotechnol. Bioprocess Eng.* 7: 340-346.
[21] Heimburger, N. and H. E. Karges (1989) Strategies to produce virus-safe blood derivatives. *Curr. Stud. Hematol. Blood Transfus.* 56: 23-33.
[22] Nowak, T., M. Niedrig, D. Bernhardt, and J. Hilfenhaus (1993) Inactivation of HIV, HBV, HCV related viruses and other viruses in human plasma derivatives by pasteurization. *Dev. Biol. Stand.* 81: 169-176.

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