Table 1. List of anti-HHV-8 seropositive samples

No.	Group	IF (TY-1)	IF (KS-1)	ELISA (Cutoff Index)
1	Healthy donor	+	+	-
2	Healthy donor	+	+/-	-
3	Healthy donor	+	+/-	-
4	Healthy donor	+	-	+ (2.41)
5	Healthy donor	+	+	-
6	Healthy donor	+	+/-	-
7	Healthy donor	+	+	+ (1.00)
8	Healthy donor	+	+/-	-
9	HBV-positive donor	+	-	-
10	HBV-positive donor	+	-	+/- (0.85)
11	HBV-positive donor	+	-	-
12	HCV-positive donor	+	-	-
13	HCV-positive donor	+	-	-
14	HCV-positive donor	+	-	-
15	TP-positive donor	+	-	-
16	TP-positive donor	+	-	-
17	TP-positive donor	+	+/-	-
18	TP-positive donor	+	-	-
19	HIV call-back donor	+	-	-
20	HIV call-back donor	+	+	-
21	HIV call-back donor	+	+	-
22	HIV call-back donor	+	-	-
23	HIV call-back donor	+	-	-
24	Multi-transfused patient	+	-	-
25	Multi-transfused patient	+	+	-
26	Multi-transfused patient	+	+	+(3.20)
27	Multi-transfused patient	+	+/-	-
28	Multi-transfused patient	+	+	-
29	Multi-transfused patient	+	+	+(1.00)

Table 2 Age and sex distribution of anti- HHV-8 seropositive donors

Age	Male	Female	Total
16 ~ 19	0/ 6	2/ 7	2/ 13
20 ~ 29	4/ 45	4/ 31	8/ 76
30 ~ 39	4/ 62	0/ 13	4/ 75
40 ~ 49	1/ 39	3/ 23	4/ 62
50 ~ 59	2/ 39	2/ 23	4/ 62
60 ~ 69	1/ 12	0/ 11	1/ 23
Total	12/ 203	11/ 108	23/ 311

positive ／ total

今回の調査で IF (TY-1)陽性を示した全 29 例の内訳を Table 1 に示した。このうち IF (KS-1)が陽性を示したのは 9 例(+/-は陰性と判定)、ELISA 法が陽性を示したのは 4 例(+/-は陰性と判定)と検査結果の乖離が認められた。

また、IF (TY-1)陽性 29 例中、頻回輸血患者を除く 23 例に関して年齢・性別を比較したところ、一定の傾向は認められなかった(Table 2)。

D. 考察

健常献血者検体において 3 法共に HHV-8 抗体陽性を示したのは 91 例中 1 例 (1.1 %) であり、頻回輸血患者検体では 64 例中 2 例 (3.1%)であった。一方、HBV 陽性献血者検体、HCV 陽性献血者検体、TP 陽性献血者検体、HIV 自己申告者検体では HHV-8 抗体 3 法共に陽性を示した検体はなかった。今回の検討において、3 法共に陽性を示した検体を真の HHV-8 抗体陽性と考えた場合、頻回輸血患者が若干高い陽性率を示したが、検体数が少なかったため、健常献血者との間に有意差は認められなかった。したがって、HHV-8 の輸血感染リスクを明らかにするために、今後 3 法陽性と判定された頻回輸血患者検体の過去の凍結保存検体を遡及調査して、輸血による感染伝播の可能性について検討する予定である。

片野らはわが国における一般健常者の HHV-8 抗体を ELISA 法でスクリーニングし、IF (TY-1)及び WB 法で確認した陽性率を 1.4%と報告している。今回の我々の結果は健常献血者における IF (TY-1)陽性率が 8.8%と高い値であったが、3 法陽性例は 1.1%と片野らの成績とほぼ同等であった。

今回、3 法の結果が不一致となった検体の中には、IF (KS-1) で弱陽性が疑われたものや、ELISA でカットオフ値より若干低いグレイゾーンと考えられる検体も含まれた。これら検査法による結果の乖離の原因として、自家製の IF 法で用いている細胞が PEL 患者由来の TY-1 であり、市販の IF 法ではカポジ肉腫患者由来の KS-1 であるという細胞株自体の違いが考えられる。使用する細胞株が異なる場合、細胞中に発現しているウイルス抗原の種類が質的、あるいは量的に異なっている可能性があり、それが抗体の反応性に影響を及ぼして乖離が生じている可能性が考えられる。また、市販 ELISA の固相抗原であるリコンビナント蛋白 ORF59 は、TY-1 株由来の cDNA からイムノスクリーニングでカポジ肉腫患者血清と強く反応したクローンであり、HHV-8 のウイルス構造蛋白をコードしている。しかし、他のクローン由来のリコンビナント蛋白 (ORF65, ORF73) を用いた ELISA の方がより多くの HHV-8 抗体を検出できることが報告されている。今後、乖離検体については、その特異性を中和試験や WB 法などにより確認を行う予定である。

また、今回の献血者における IF (TY-1)陽性例を年齢・性別で比較した結果では、一定の傾向は認められなかった。通常、EBV や CMV 等のヘルペスウイルスでは加齢とともにウイルス抗体陽性率が高くなるが、HHV-8 についてはそのような傾向は認められなかった。その理由は不明であるが、わが国における HHV-8 の浸淫度が低いため感染伝播が起こりにくいことや、感染経路の違いなどが影響している可能性がある。

E. 結論

健常献血者検体における HHV-8 抗体 3 法陽性率は 1.1 %、頻回輸血患者検体における HHV-8 抗体 3

法陽性率は 3.1%、HBV 陽性献血者検体、HCV 陽性献血者検体、TP 陽性献血者検体、HIV 自己申告者検体では HHV-8 抗体 3 法陽性率は 0%であった。頻回輸血患者検体で若干高い HHV-8 抗体陽性率を示したが、今回の調査では検査数が少ないため有意差を認めることはできなかった。今後は既感染のマーカー検出法として何が最適な方法かを検討するためにも、乖離原因を明らかにする必要があると思われる。その上で輸血感染リスクの研究を進めていかなければならない。

F. 研究発表

該当なし

G. 知的所有権の取得状況

該当なし

Guidance Document on Viral Inactivation and Removal Procedures Intended to Assure the Viral Safety of Blood Plasma Products

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Listing of Figures

I. INTRODUCTION AND SCOPE

This viral validation guidance pertains to the validation and assessment of viral inactivation and removal steps employed in the manufacture of blood plasma derivatives and virally inactivated plasma for transfusion prepared from plasma pools or from individual donations. It does not address donor selection or blood screening procedures in any detail although it is recognized that both contribute to the safety profile of the manufactured products. This guidance is being provided to assist national control authorities and manufacturers less familiar with viral decontamination processes to assess compliance with applicable regulations and existing guidelines. Data are provided for illustrative purposes only and do not necessarily represent limits.

Abstracted principles taken from published guidances

This guidance is intended to amplify and not replace guidance documents published by established regulatory authorities, including the European Committee on Proprietary Medicinal Products (CPMP)[1, 2], the U.S. Food and Drug Administration (FDA)[3], and the Japanese Ministry of Health and Welfare[4]. Some of the principles espoused in these guidances that relate to viral inactivation and removal follow.

- Viral safety derives from 3 complementary approaches during manufacture; i.e., donor selection, testing of donations and plasma pools, and viral inactivation and removal in the course of manufacture, each of which requires strict adherence to GMP's. Although this Guidance addresses only the third of these, no approach provides a sufficient level of assurance alone, and safety will only be achieved using a combination of the three.
- Viral contamination can arise from the donor, or, less commonly, from other sources introduced during manufacture, such as from reagents which are employed.
- Viral validation studies are intended to assess the degree to which viruses are eliminated during manufacture. These studies only serve to approximate the inactivation and removal that occurs during routine manufacture since model viruses employed in the studies may differ from those present in blood, and it may be difficult or impossible to truly model the conditions employed during manufacture. Thus, the appropriateness of the studies needs to be reviewed on a case-by-case basis, with the manufacturer justifying the viruses chosen and the validation conditions employed.
- Viral removal should be distinguished from viral inactivation. This is important in ensuring the accurate modeling of a process step and identifying the process parameters which have the greatest effect on reducing infectivity. For example, if a chromatography step removes virus, flow rates and column dimensions are important process variables, whereas if the buffer used inactivates virus, temperatures and pH are likely to be more significant.

- Viruses to be studied where required should include: HIV-1; a model for hepatitis C such as Sindbis virus or bovine viral diarrhea virus (BVDV); one or more non-enveloped viruses such as hepatitis A virus, encephalomyocarditis virus (EMCV), or porcine parvovirus; and an enveloped DNA virus such as pseudorabies virus or duck hepatitis B virus.
- The ability of a process to inactivate or remove virus should take into account (a) the reduction in virus titer achieved, (b) the rate of inactivation and the shape of the inactivation curves, (c) how robust the step is to changes in process conditions, and (d) the selectivity of the process for viruses of different classes. Data should be analyzed using appropriate statistical procedures.
- Purification procedures such as precipitation or chromatography can contribute to virus removal; however, removal depends critically on the protein composition and the separation conditions used, and partition processes are difficult to scale down for validation purposes. Therefore, all appropriate specifications and accepted tolerances should be stated, and control data provided. For columns and chromatographic media, the conditions of storage, preservation, and regeneration should be described.
- Validation studies need to be well documented to ensure proper execution of the procedure. The highest titer of virus that reasonably can be employed should be added (spiked) into the solution to be tested at a ratio not to exceed 1 part of virus to 9 parts sample. Virus infectivity start titer should be measured most preferably after addition to the sample and then with time during the virus inactivation and removal procedure. Worst case conditions must be studied. Appropriate controls should be run to demonstrate validity and sensitivity of the assay.
- All viral infectivity tests suffer from the limitation that the ability to detect low viral concentrations depends for statistical reasons on the size of the sample. Consequently, the largest sample size that can be practically assayed should be assayed if the study indicates that all virus is inactivated or removed.
- Procedures should be employed through the course of manufacturing to prevent recontamination following use of a virus inactivation or removal method.
- Priority for validating viral inactivation steps used in the manufacture of plasma protein solutions should be given to those products with the highest risk potential. The priority order is, starting from the highest, coagulation factors, proteolytic inhibitors, intravenous immunoglobulins, intramuscular immunoglobulins, and albumin.

II. VIRAL BURDEN

Medicinal products made from human blood include clotting factors, immunoglobulins and albumin among others, all of which at some time have transmitted serious virus infections to recipients. The object of viral inactivation/removal procedures is to

improve viral safety so that transmissions no longer occur. Viruses of particular concern include hepatitis B virus (HBV), hepatitis C virus (HCV) and human immunodeficiency virus (HIV), all of which have been transmitted by products from human blood, and all of which cause life threatening diseases. Other viruses of concern include hepatitis A virus and parvovirus B19, both of which have been transmitted by clotting factor concentrates. The importance of a virus may depend on the patient group. For example parvovirus B19 infects the red blood cell precursors and effectively eliminates them for a period; in most cases this is relatively mild in its effects because there is a substantial buffer of mature red cells, but in patients with hemolytic anemias (such as sickle cell anemia), parvovirus infections can be fatal because the life span of mature red cells is shorter. Parvovirus B19 may be of greater concern in Africa where sickle cell anemia is relatively more common than in Europe, and it is possible that other agents (e.g., hepatitis E virus) would be significant in other geographical settings depending on their prevalence in the donor population. Certain viruses such as cytomegalovirus and human T lymphotropic virus I and II (HTLV I+II) are strongly cell associated and therefore not considered to pose a significant risk in therapeutic proteins derived from human blood, although they have been transmitted by cellular components in blood transfusions. Bacteria and parasitic infections including malaria and trypanosomes are not a risk with plasma products which have been sterile filtered with a 0.2 µm filter.

If the product is to be safe, the production process should be able to inactivate and/or remove the viruses with which it will be challenged. This in turn depends on the number of infected donors contributing to the pooled starting material and the titer of infectious virus in those donations. Prior to implementation of screening tests, estimates of the frequency of occurrence in the U.S. or European donor populations of hepatitis viruses, HIV and parvovirus and their titers are given in Figure II-1. For example, prior to developing tests for HCV antibody, approximately 1-2% of donors were unknowingly infected with HCV. Another example is parvovirus, which we now know is present in 1/1,000 – 1/7,000 blood donors, largely because it is a common infection in the general population and tests are not routinely employed. When this information is coupled with the titer (concentration) of virus in contaminated units and the number of donors contributing to the plasma pool, the titer in the plasma pool can be calculated (Figure II-1.) Since titers of HCV RNA in an infected individual may range from 10^4 to 10^8 genome equivalents (GE)/ml and of parvovirus B19 DNA from 10^2 to 10^{12} GE/ml, plasma pools would be expected to contain $10^2 – 10^4$ GE/mL of HCV and 0 – 10^9 GE/mL of parvovirus. More simply put, most pools of 10,000 or more unscreened donor units would be expected to be contaminated with HCV and parvovirus, while contamination with HBV, HIV and HAV would occur at a lower frequency. Experience bears out these predictions. As an example, in one study conducted at the National Institute for Biological Standards and Control (NIBSC) on samples collected prior to implementing HCV antibody testing, 8/8 plasma pools tested positive for HCV RNA, and three had titers of 10^1 GE/mL when subjected to limiting dilution titration. Other studies show that while many plasma pools have only low levels of parvovirus B19 DNA, some have levels that exceed 10^7 GE/mL. It should be noted that the prevalence of virally

infected units depends on several factors including the population from which donors are drawn and, for parvovirus, seasonal variations.

Figure II-1. Viruses in Plasma From Unscreened Donor Blood

Virus	Prevalence in Donor Blood	Viral Titer (GE/mL)	Calculated Titer in Plasma Pool (GE/mL)*
Hepatitis B	1/10,000	10^3 - 10^8	0 - 10^4
Hepatitis C	1/50 - 1/100	10^4 - 10^6	10^2 - 10^4
HIV	1/1,000 – 1/10,000	10^3 - 10^7	0 - 10^4
Hepatitis A	1/500,000	10^3 - 10^5	0 - 10^1
Parvovirus B19	1/1,000 – 1/7,000	10^2 - 10^{12}	0 – 10^9

* Assumes the pooling of 10,000 units

A study conducted at the Paul-Ehrlich-Institute shows the frequency of HCV RNA positive pools before screening of donors and after screening was introduced using first or second generation tests for HCV antibody (Figure II-2). It is important to note that the effect of screening was to reduce the number of positive pools, but not the titer in those pools which were contaminated. This is a consequence of using a test for antibody rather than for virus and, in the case of HCV and many other viruses, that peak titers occur prior to antibody appearance in the circulation.

Figure II-2. Frequency of HCV RNA positive plasma pools following testing of single donations for anti-HCV antibody[5].

Screening test	Number of pools (positive/total)	Percent HCV PCR positive
None	8/8	100%
1st generation antibody test	65/85	76%
2nd generation antibody test (US)	46/88	52%
2nd generation antibody test (Europe)	3/35	8%

Since screening of donors for markers of infection such as hepatitis B surface antigen or antibodies to HIV or HCV can reduce the number of positive pools and, in certain circumstances, the virus load in the starting material, screening is an important element in assuring viral safety. Several reasons serve to explain why plasma pools continue to be contaminated despite use of sensitive donor screening tests. The most important is that there is a period between infection and the development of an antibody response

(the window period) where a donation will be infectious but not detectable. Nucleic acid amplification technology (NAT) has been introduced in some instances to detect viral nucleic acid. As nucleic acid is associated with the virus itself rather than the host response to infection, NAT minimizes the window period and reduces the total quantity of virus in the plasma pool. Nonetheless, all screening methods are subject to the criticism that they are unable to detect virus infection below a certain level, and errors in the screening process may also occur, particularly where large numbers of donations are used. Additionally, screening is limited to the viruses being tested for. Thus while screening helps to ensure that the virus load is kept to a minimum, it is not sufficient to ensure safety alone, and the ability of the production process to remove or inactivate virus is a crucial second element. The proportion of potential donors infected will depend on the particular region. In certain areas, HBV or HIV infections may be far more common than in donors from countries where the strategies for ensuring viral safety have evolved. Where this is the case the ability of the production process to inactivate or remove viruses will be even more important.

In practice many processes give a product which is safe. The ability of a process to remove or inactivate virus infectivity is conventionally expressed in terms of the logarithm of the reduction in infectivity reported. Total infectivity is calculated as the infectious titer (infectious units per ml) multiplied by the volume. In the context of bacteria, a sterile product has been defined conventionally as one having fewer than one infectious organism in one million doses. No figure has been agreed upon for viral sterility because viruses are more difficult to assay in the final product, the titer of virus in the stocks used to spike product is limited, and assessing the ability of a process to remove or inactivate viruses is subject to significant sources of error.

The ability of a process to remove or inactivate viruses is measured on a laboratory scale and not in the production facility where it would be inappropriate to introduce infectious virus deliberately. The difficulties of establishing an adequate laboratory model mean that the figures produced are unlikely to fully reflect manufacturing operations. In addition data will be required for the particular process as slight differences in process parameters (such as temperature or moisture content) can have a major effect on the clearance observed. In general for a product to be safe the process must remove or inactivate virus infectivity to a much greater extent than the level of virus in the starting materials. As part of the philosophy that the process must have excess capacity with respect to virus reduction, some regulatory authorities have suggested that the overall manufacturing process should have at least two defined stages able to remove or inactivate virus effectively and by different mechanisms (e.g. inactivation by chemical treatment followed by some robust physical removal step). This suggestion may be especially important where the population of donors contributing to the plasma pool has a high incidence of blood borne viruses, leading to a very high viral load in the material being processed, or when single donor units are virally inactivated and then transfused without pooling. A second value in employing two complementary methods of virus inactivation and removal is the potential to increase the spectrum of viruses covered.

Prions, the putative causative agent of the transmissible spongiform encephalopathies including Creutzfeld Jakob Disease of humans, are a matter of concern, especially as a result of the occurrence of variant CJD in the United Kingdom following the epidemic of bovine spongiform encephalopathy.[6] There is no evidence that CJD has been transmitted by blood or products derived from it in clinical practice, but there is ongoing concern stemming in part from experimental evidence in animal models that infectivity could be present in blood, albeit late in infection and at low levels. As compared with these animal data, some comfort can be derived from the fact that there has not been an increase in the incidence of classic CJD, one death per million head of population per year wherever it has been measured, despite the increased transfusion of blood and the extremely hardy nature of the agent. Nonetheless, in addition to the disease occurring in an unpredictable sporadic form, since it also occurs in certain populations at high frequency, individuals known to be at risk (i.e. those genetically predisposed as defined by one or more cases of CJD in the family, or those having been exposed to human dura mater or other materials derived from human brain) are excluded from donation in most countries. The nature of the agent makes it difficult to destroy, although it may be physically removed in the course of production. In the absence of a diagnostic test, precautions are solely based on donor exclusion.

III. REVIEW OF WELL RECOGNIZED VIRAL INACTIVATION/REMOVAL METHODS

The methods described in this section are generally recognized as contributing substantially to viral safety based on (a) their application to a variety of products, (b) use by several manufacturers, and (c) the availability of a substantial body of pre-clinical and clinical information. Well recognized methods of inactivation (pasteurization, dry heat, vapor heat, solvent/detergent, and low pH are described in Section A, and well recognized methods of removal (precipitation, chromatography, nanofiltration) are described in Section B. The selection of the viral inactivation and removal methods to be employed depends on the size and lability of the protein being prepared, the method(s) of purification the manufacturer wishes to use, and the nature and titer of viruses which are of concern. Each method of inactivation and removal has special characteristics which need to be taken into account. As examples, solvent/detergent is very effective against enveloped viruses but does not inactivate non-enveloped viruses. If HBV is a principal concern, solvent/detergent may have an advantage over methods of heating since HBV is known to be relatively heat stable. On the other hand, several methods of heating have been shown to inactivate 4 logs or more of HAV, and where HAV is considered to be an issue, heat has an advantage over solvent/detergent. From a virus safety perspective, the best procedures will use a combination of methods that are complementary to one another, consistent with the maintenance of protein structure and function. Combinations have the advantage of increasing the spectrum of virus covered as well as increasing the total quantity of virus that is eliminated. The use of two complementary methods of virus inactivation and removal may be especially important in regions where viral loads present in plasma pools are relatively high,

perhaps because viral screening tests are only partially deployed. In addition to viral safety, the maintenance of protein structure and function is equally important and must be evaluated thoroughly. Most viral inactivation and removal methods have not stimulated an antibody response on infusion of the product into man, however, as a cautionary note, there are two documented instances (one involving a pasteurized product and the other involving a product treated with solvent/detergent combined with pasteurization) where treated product was found to be immunogenic.

Regardless of the methods employed, strict adherence to good manufacturing practices (GMP's) is required if viral safety is to be achieved. Equipment and processes need to be validated, employed methods need to be described adequately, personnel need to be trained, actions taken need to be documented, deviations need to be recorded and investigated, and a quality assurance unit that reviews the design and implementation of processes as well as final production records needs to be established. Additionally, facility layout and the flow of people, product and waste contribute substantially to the overall safety of the product. To avoid cross contamination, the best implementations provide for unidirectional flow of product through the course of the manufacturing scheme with a clear separation at steps shown to contribute substantially to virus inactivation and removal.

A. *Methods of Inactivation*

1. Pasteurization of albumin

Albumin solutions are heated as a liquid at 60±0.5°C for 10-11 continuous hours, most typically following sterile filtration and dispensing into final container glass vials. To prevent albumin denaturation, low concentrations of sodium caprylate by itself or with N-acetyl tryptophan are added prior to sterile filtration. Safety with respect to hepatitis viruses and HIV has been demonstrated for decades, with the exception of but few cases[7]. The inactivation of model viruses added to 5% albumin solution on heating at 60°C is shown in Figure IIIA-1. Infectious virus can no longer be detected after 10 minutes treatment. Because the conditions of treatment are well established and, in some countries, specified by regulation, manufacturers are not required to validate the effectiveness of the treatment itself; however, manufacturers must demonstrate that the process parameters of temperature and time are met. Temperature homogeneity is typically achieved by total immersion of the vials in a water bath or by placing the vials in a forced air oven. In both cases temperature mapping studies are required to demonstrate homogeneity, measuring both the temperature of the water or air as well as of the product itself. These studies must be performed with representative loads. Once validated, temperature probes are placed at strategic points in the water bath or oven during each pasteurization run.

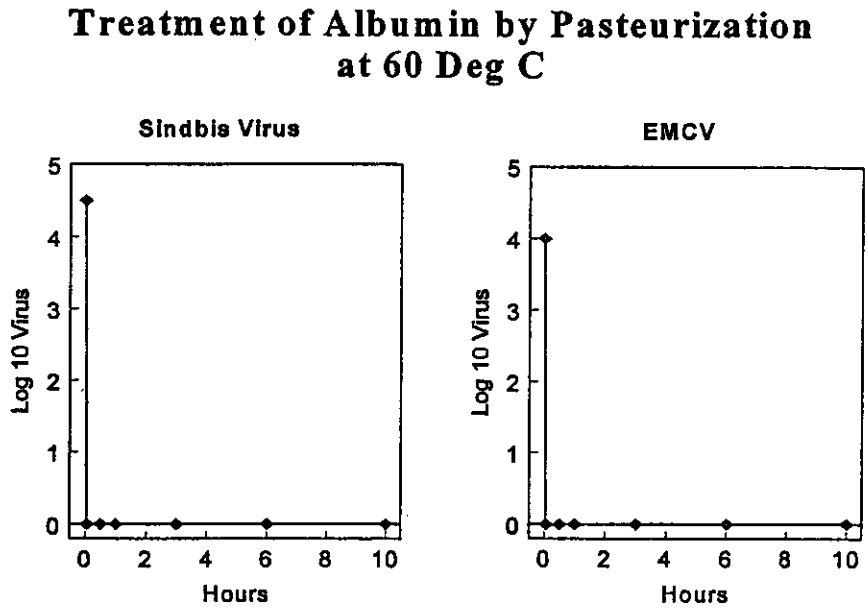


Figure IIIA-1. Rate of virus inactivation on pasteurization of 5% albumin.
From Horowitz et al., Transfusion 1985; 25: 523-527.

2. Pasteurization of other protein solutions

Most proteins denature when heated at 60°C in the liquid state. In order to maintain the biological function of the more labile proteins, general stabilizers like amino acids, sugars, or citrate are added. Since these also stabilize viruses, virus inactivation must be validated in model studies for each product under the conditions of treatment specified by the manufacturer. Pasteurization has been used successfully with both coagulation factors and immune globulin solutions, although in rare instances HBV has been reported to be transmitted.[8] A common method of preparing factor VIII is to heat it at 60°C for 10 hours in the presence of high concentrations of glycine and sucrose or selected salts. Published results showing the extent and rate of virus inactivation with AHF are given in Figures IIIA-2 and IIIA-3.

Prior to heating, the solution is typically filtered through a 1 micrometer or finer filter to eliminate particles that might entrap and further stabilize virus. Heating is conducted in a jacketed tank with the solution typically stirred throughout the heat cycle. Temperature mapping studies are conducted to ensure that all points in the tank are within the range specified by the process record. Care must be taken to ensure that all parts of the tank, including the lid where solution might splash, are heated. Viral inactivation studies, conducted under worst case conditions, are performed at the coldest temperature that might be encountered in an acceptable production run. Protein recovery should be monitored during virus inactivation studies and should be comparable to that achieved at scale.

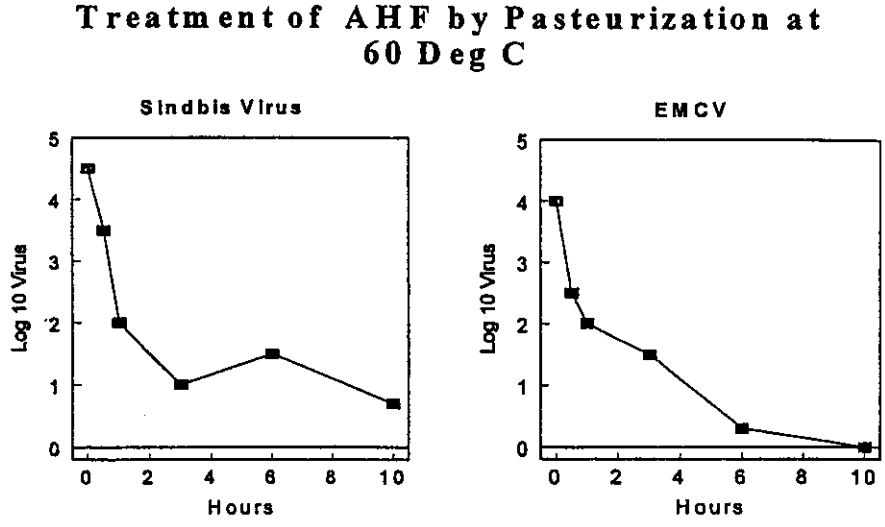


Figure IIIA-2. Rate of virus inactivation on pasteurization of AHF in the presence of 50% sucrose and 2.75 M glycine.

From Horowitz et al., Transfusion 1985; 25: 523-527.

Figure IIIA-3. Treatment of a Factor VIII Solution by Pasteurization.

Virus	Extent of Inactivation (log ID_{50})	Inactivation Time (hours required)
HIV	≥5.0	1.0
CMV	≥6.0	8
EBV	≥3.3	0.5
HSV	≥5.9	4
Poliovirus	≥7.1	10
Vaccinia virus	6.2	10

From Hilfenhaus, J., et al., Vox Sang, 1986; 50: 208-11.

3. Heating of dry (lyophilized) products

Proteins can withstand being heated at 60-80°C or higher when they are first lyophilized to remove water. Heating at 60-68°C for times up to 72 hours generally has not eliminated hepatitis transmission[Colombo, 1985 #320], whereas heating at 80°C has produced favorable results with respect to HBV, HCV, HIV, and HAV.[9] Since viruses are also more stable following lyophilization, virus inactivation must be validated for each product under the conditions of treatment specified by the manufacturer. Viral inactivation is influenced by residual moisture, the formulation (e.g., content of protein, sugars, salts, amino acids), and by the freezing and lyophilization cycles. Residual moisture is influenced by the lyophilization cycle and may be introduced inadvertently via the rubber stoppers.

Since virus inactivation is very sensitive to residual moisture content, the setting of upper and lower moisture limits should be based on viral validation studies, and the variation of moisture content between vials should be within the limits set. To ensure reproducibility, one manufacturer, during the freeze drying, has stipulated that the temperature in three or more product vials together with the shelf coolant temperature and chamber pressure must remain within defined limits for each timed phase of the lyophilization cycle for every manufactured batch. Following freeze drying, vials are stoppered under sterile, dry nitrogen at atmospheric pressure to ensure a constant atmosphere from vial-to-vial during dry heat treatment. In addition, from every lyophilization run, the residual moisture content of 5 vials out of a lot of 1,500 is measured following heat treatment. The moisture values of these vials are used to calculate the 95% confidence interval for the batch, and this interval must be within the upper and lower limits of moisture defined for the product.

Again using the specifications of one manufacturer, the dry heat treatment, itself, is performed at 80.25±0.75°C for 72 hours. Process monitoring during heat treatment is carried out by means of temperature sensors located in 10 vials distributed throughout the load and 2 "air" probes located at the previously determined warmest and coldest points in the oven. All temperature sensors (both probed vials and those measuring air temperature) must reach +79.5°C before the cycle timer starts. All temperature sensors must remain stable between +79.5°C and 81°C for a continuous period of 72 hours. In addition, the dry heat ovens are validated at pre-determined 6 monthly intervals, when a further independent 12 probes (10 vials and 2 air) linked to a separate chart recorder are included to increase the temperature point coverage to 24. In this way the temperature control is tested and the temperature spread within the cabinets established. Cycle time on the automatic control is also checked for accuracy.

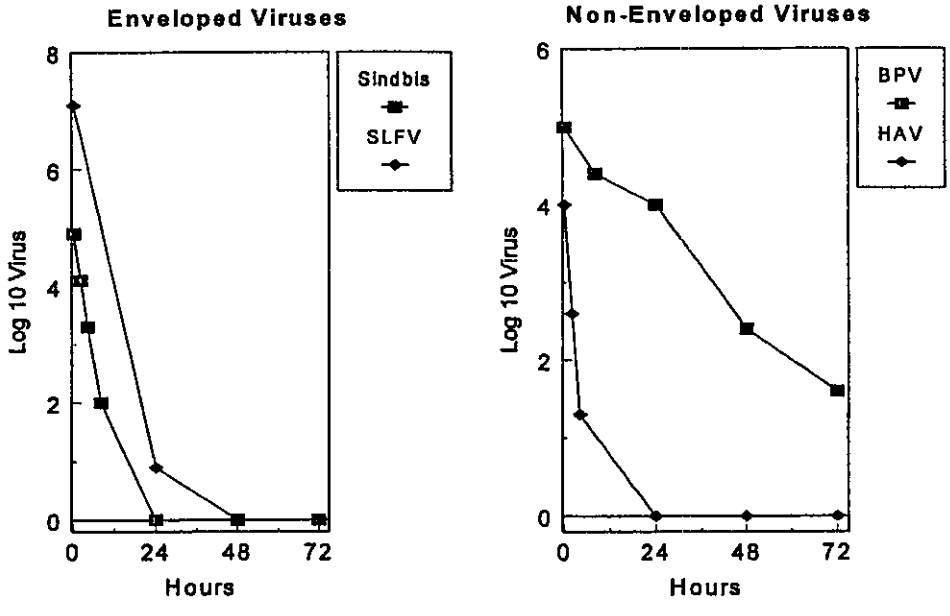
Typical results achieved on heating factor VIII at 80°C are given in Figure IIIA-4 and IIIA-5.

Figure IIIA-4. Treatment of Lyophilized Factor VIII at 80°C for 72 Hours.

Virus	Extent of Inactivation (log ID_{50})	Inactivation Time (hours required)
Sindbis virus	8	72
HIV	≥6.4	72
Vaccinia virus	2.6 - 3.3	72
HSV	2.2	48
SLFV	≥6.9	24
HAV	≥4.3	24
CPV	≥2.1	48

From Knevelman A., et al., Vox Sang 1994; 66: 89-95; Winkelman L et al. Curr Stud Hematol Blood Transfus. 1989; 56: 55-69; and Hart HF, et al. Vox Sang 1994; 67: 345-50.

Figure IIIA-5. Rate of inactivation of enveloped and non-enveloped viruses on dry heat treatment of AHF.



SLFV, Semliki Forest virus; BPV, bovine parvovirus; HAV, hepatitis A virus. Results graciously provided by the Scottish National Blood Transfusion Service.

4. Heating of lyophilized products under humidified conditions

("vapor heated")

At equivalent temperatures, a higher level of virus inactivation can be achieved by the addition of water vapor before initiating the heat cycle. To assure proper application of this approach, the material to be heated, the addition of moisture, and the heat cycle need to be tightly controlled. In one implementation, freeze-dried intermediate bulk product is homogenized by a combination of sieving and milling. After determining the residual water content, the freeze-dried intermediate is transferred into a stainless steel tank where an amount of water vapor, pre-determined based on the weight and the residual water content of the lyophilized product, is slowly added to adjust the water content to 7 - 8% (w/w). After an equilibration period, the water content is measured again before the product is ready for vapor heating. The intermediate product is transferred to a stainless steel cylinder. The cylinder is flushed with dry nitrogen to remove oxygen, and a pressure test is performed to ensure that the cylinder is airtight. This cylinder is then brought into a heating cabinet equipped with electric heating and a fan to ensure even temperature distribution. The intermediate product within the cylinder is heated according to the temperature regimen specified for the particular product. The cylinder is subjected to an oscillating rotation, changing direction every half turn, until the end of vapor heating. During the heating process the pressure inside the vessel rises due to heating of the enclosed nitrogen, which cannot expand in the closed cylinder, and also due to evaporating water vapor from the moist intermediate product. After vapor heating, the heating cabinet is opened from the other side, and the product is further processed in a different and isolated manufacturing zone to prevent cross-contamination from non-inactivated product.

To assure consistency from lot-to-lot, ranges for protein, salt and water content are set based on preliminary viral infectivity and protein functional studies. Additionally, the ratio of product weight to cylinder volume is specified for each product. A pressure test is performed before start of vapor heating to ensure that the cylinder is airtight. During heating, product and air temperature (one temperature sensor each) and pressure within the cylinder are measured continuously and must conform to pre-set specifications set for each. Following vapor heating, the water content of the intermediate is measured again.

Clinical safety with respect to hepatitis viruses and HIV has been demonstrated.[10] Typical results achieved on heating several products are given in Figure IIIA-6 and IIIA-7.

Figure IIIA-6. One Step Vapor Heating at 60°C for 10 hours

Product	Virus	Extent of Inactivation (log ID_{50})	Inactivation Time (hours required)
Intermediate purity factor VIII	HAV	>5.9	8
	HIV	>6.8	10
	PRV	5.9	10
High purity factor VIII	HAV	5.9	10
	HIV	6.7	10
	PRV	5.6	10

Data and process information provided courtesy of Baxter/Immuno. See also Barrett *et al.* Transfusion: 1997; 37: 215-220 and Dorner F. and Barrett N. Hämostaseologie 1996; 16: 282-285.

Figure IIIA-7. Two Step Vapor Heating at 60°C for 10 Hours and at 80°C for 1 Hour

Product	Virus	Extent of Inactivation (log ID_{50})	Inactivation Time (hours required)
Intermediate purity factor IX	HAV	>5.7	6
	HIV	>6.5	6
	PRV	>7.1	8
High purity factor IX	HAV	>6.7	3
	HIV	>7.9	8
	PRV	>6.8	8

Data and process information provided courtesy of Baxter/Immuno. Historically, the 60°C and the 80°C heating steps were validated individually, summing the two reduction factors (RF). The values given above reflect the inactivation capacity of the procedure according to current thinking, i.e. without adding *RF* values of steps based on the same mechanism of inactivation.

See also Barrett *et al.* Transfusion 1997; 37: 215-220 and Dorner, F. and Barrett, N. Hämostaseologie 1996; 16: 282-285.

5. Solvent/Detergent treatment

Organic solvent/detergent mixtures disrupt the lipid membrane of enveloped viruses. Once disrupted, the virus no longer can bind to and infect cells. Non-enveloped viruses are not inactivated. Typical conditions which are used are 0.3% tri(n-butyl) phosphate (TNBP) and 1% nonionic detergent, either Tween 80 or Triton X-100, at 24°C for a minimum of 4 hours with Triton X-100 or 6 hours with Tween 80. When using TNBP/Triton X-100, some preparations can be treated successfully at 4°C. The final selection of treatment conditions must be based on viral validation studies, testing the worst case conditions; i.e., lowest permitted temperature and reagent concentration, highest permitted product concentration. Prior to treatment, solutions are filtered through a 1 micrometer filter to eliminate virus entrapped in particles. If filtration is performed after addition of the reagents, the filtration process should be demonstrated to not alter the levels of the added solvent and detergent. The solution is stirred gently throughout the incubation period. Physical validation should confirm that mixing achieves a homogeneous solution and that the target temperature is maintained throughout the designated incubation period. Mixing homogeneity is best verified by measuring TNBP or detergent concentrations at different locations within the tank, although measuring dye distribution might be an acceptable substitute. To ensure that every droplet containing virus is contacted by the reagents, an initial incubation for 30-60 minutes is typically conducted in one tank after which the solution is transferred into a second tank where the remainder of the incubation is conducted. In this manner, any droplet on the lid or surface of the first tank which might not be contacted with the SD reagents is excluded. The use of a static mixer where reagents and plasma product are premixed prior to entrance into the tank is an acceptable alternate. The tank in which viral inactivation is completed is located in a separate room in order to limit the opportunity for post-treatment contamination. This room typically has its own dedicated equipment and may have its own air supply.

When performing viral validations, the reaction is stopped either by dilution or, in some cases, adsorption of the TNBP and Triton X-100 by a C18 hydrophobic resin. An appropriate control must be run establishing that virus inactivation is not on-going following use of the stop procedure. Safety with respect to HBV, HCV and HIV has been demonstrated in numerous clinical studies which reflect the high level of virus inactivation demonstrated in both laboratory and chimpanzee studies. Typical results achieved on treating an AHF concentrate and fibrinogen at 24°C are given in Figures IIIA-8 and IIIA-9.