Part 2, Red Blood Cells and Platelets

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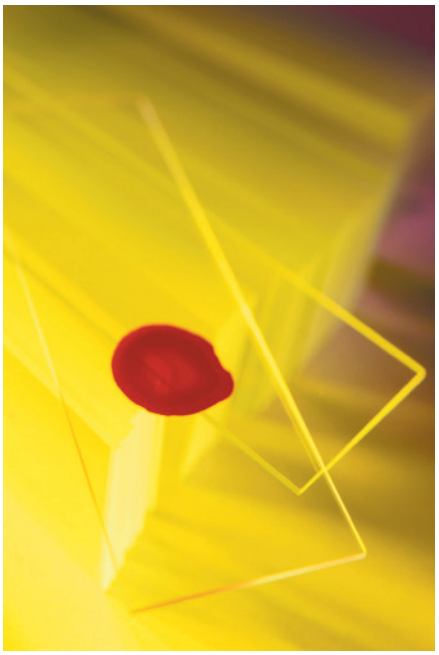

Gail Sofer

Patient safety requires viral clearance (inactivation or removal). Surveying the literature on the various methods (both successful and unsuccessful) used to achieve that goal can help meet those requirements. Part 1 in this series dealt with skin, bones, and cells other than platelets or red blood cells (1). In Part 2, the series covers red blood cells and platelets.

Virus inactivation methods reported for platelet and red blood cell (RBC) concentrates include photochemical treatments using psoralens and dyes, Inactine, and anchor-linker effectors. Photoactive compounds include photodynamic dyes activated by visible light that act by oxygen-dependent generation of reactive molecular species and UV-activated, intercalating compounds that form covalent adducts with nucleic acids.

Psoralens are activated at wavelengths in which hemoglobin absorbs (UVA 300–400 nm) and interferes with their activation, so they are not particularly useful for RBCs. Dyes that bind with viruses and are activated with light are used in virus inactivation, but they can have negative effects on cells. Photodynamic methods can cause platelet injury. After photoinactivation, the damage to RBC properties can increase during storage. A 1991 review of inactivation of viruses associated with cellular components provides references from the 1980s and also addresses carcinogenicity, neoimmunogens, and the cost of some of the inactivation methods (2).

Red Blood Cells

Table 1 summarizes the various methods used for virus inactivation in RBCs, details the virus inactivated by each method, and references the literature that discusses each procedure.

Phenothiazine dyes. Methylene blue (MB) and dimethymethylene blue (DMMB) are photosensitizing phenothiazine dyes. MB targets nucleic acid and protein and is activated by light at a wavelength of 630 nm. DMMB targets nucleic acid and is activated by light in the 400–1,000 nm range (3). MB can only inactivate extracellular viruses. DMMB phototreatment can inactivate several intracellular and extracellular model viruses with minimal alteration of the RBCs during 42 days of storage at 1–6°C. RBC suspensions were treated with cool white fluorescent light and four µmol per liter of DMMB. Table 2 shows $\log_{10}$ reduction values from this process (4).

A comparison of MB and methylene violet (MV) showed that MV can inactivate intracellular as well as extracellular viruses. Under conditions in which both MB and MV inactivated approximately five $\log_{10}$ extracellular VSV, MV provided greater than four $\log_{10}$ reduction of intracellular VSV, whereas MB inactivated only 0.88 $\log_{10}$ of the intracellular virus (5).

In another study of virus inactivation using MB photosensitization, conditions that inactivated greater than six $\log_{10}$ of VSV failed to inactivate EMC. However, less stringent phototreatment conditions inactivated five $\log_{10}$ of SIN and four $\log_{10}$ of extracellular HIV-1. Intracellular HIV was not inactivated under the conditions evaluated (6).

VSV and DHBV were rapidly photoinactivated by DMMB. Inactivation depended on the DMMB concentration and red light dose. DMMB without light was ineffective. Inactivation of VSV required a DMMB concentration 100-fold greater than that required for DHBV at the same light exposure. Micromolar concentrations of DMMB inactivated the DHBV virus within seconds (7).

Phthalocyanines are blue dyes with a maximum absorption in the 600–700 nm (red light) range. They have been used to inactivate lipid-enveloped viruses in RBC concentrates. When silicon phthalocyanine Pc4 was added to RBCs and exposed to red light, greater than five $\log_{10}$ of VSV were inactivated (8). Sulfophthalocyanines have been shown to inactivate vaccinia in an RBC suspension (9). Irradiation of RBCs containing phthalocyanines with red light for 30 minutes was shown to completely inactivate VSV added to the RBCs (10). To protect the cells, a water-soluble analogue of vitamin E (Trolox C) was added (11). Increasing the light irradiance damaged the RBCs but did not change the VSV inactivation (12).

In another study, quenchers were added to prevent cellular damage and to increase specificity of the viruses killed by aluminum phthalocyanine tetrasulfonate ($AlPcS_4$) and light. VSV was rapidly and completely inactivated (13). RBC concentrates, whole blood, and platelets were treated with $AlPcS_4$ and activated with light. That treatment inactivated more than 5.5 $\log_{10}$ cell-free VSV, more than 5.6 $\log_{10}$ cell-associated VSV, and more than 4.7 $\log_{10}$ cell-associated SIN. Inactivation of cell-free HIV was 4.2 $\log_{10}$, and cell-associated HIV inactivation was 3.6 $\log_{10}$. As expected, EMC (a nonenveloped virus) was not inactivated (14,15).

Merocyanine 540. Inactivation by merocyanine 540 has also been proposed (16,17). But as noted in a review article (18), albumin inhibits its ability to make enveloped viruses sensitive to photoinactivation, and its action spectrum overlaps with that of hemoglobin at 570 nm.

Porphyrin derivatives. Benzoporphyrin derivative ring A, activated with red light, was effective in eliminating free viruses and virally infected cells from spiked red cell concentrates. VSV and FeLV were used as model viruses, and the red cell membranes seemed to be undamaged (19).

HSV-1 was used for screening photoactive compounds, then effective compounds were challenged with HIV-1, CMV, and SIV in both culture medium and blood. The RBCs were undamaged. The most effective photoactive dyes were dihematoporphyrin ether (DHE), hematoporphyrin derivative (HPD), benzoporphyrin derivatives (BPD), and an alkyl-substituted sapphyrin (20).

Table 1. A summary of viral inactivation methods for red blood cells that have appeared in the literature since 1990

Viruses	Method	References
VSV, SIN, HIV	Aluminum phthalocyanine tetrasulfonates	13–15
VSV, FeLV	Benzoporphyrin derivative	19
HIV	Hypericin	21
PRV, BVDV, VSV, SV-40, PPV	Inactine	22
VSV, SIN, HIV	Merocyanine 540	16,17
VSV, DHBV	Phenothiazine dyes	7
VSV, PRV	Phenothiazine dyes	4,5
VSV, SIN, HIV	Phenothiazine dyes	6
VSV	Phthalocyanines	10,12
HIV-1, CMV, SIV	Porphyrin derivatives, sapphyrin	20
HIV, DHBV, BVDV	S-303	23
VSV	Silicon phthalocyanines	42
Vaccinia	Sulfophthalocyanines	9

Table 2. Log_{10} reduction values for red blood cells treated with four μmol/L of dimethymethylene blue and cool white fluorescent light

Extracellular Viruses	Intracellular Viruses	Log_{10} Reduction
VSV		>4.4
	VSV	>3.0
PRV		>5.0
	PRV	>4.8
EMC		0.06
BVDV		>4.7

Other methods. For most blood components, *hypericin* caused no adverse response and completely inactivated 10^6 $TCID_{50}$ of HIV in diluted packed red cells after illumination with fluorescent light for one hour (21).

Inactine (PEN 110) provided reduction greater than five log_{10} of both the enveloped viruses (PRV, BVDV, VSV) and the nonenveloped viruses (SV-40 and PPV) in human RBC concentrates (22).

Frangible anchor-linked effectors (FRALEs) are activated by a shift in pH after they have been added to packed red blood cells. FRALEs target nucleic acids and form covalent adducts. Compound S-303 has been shown to inactivate high titers of cell-free and cell-associated HIV, DHBV, VSV, HSV, and BVDV (23). Inactivation does not require light.

Platelets

Table 3 lists the various methods described in the literature for inactivating virus in platelets.

Phthalocyanines. The data presented for virus inactivation using phthalocyanines in infected RBCs also apply to virus inactivation in platelets (14). $AlPcS_4$ and light treatment resulted in inactivation of more than 5.5 log_{10} of cell-free VSV, more than 5.6 log_{10} of cell-associated VSV, and more than 4.7 log_{10} of cell-associated SIN. Cell-free HIV was inactivated by 4.2 log_{10}, and cell-associated HIV was inactivated by 3.6 log_{10}.

Psoralens. Psoralen S-59 (150 μM) and UVA (320–400 nm) treatment of platelet concentrates derived from buffy coat inactivated cell-associated HIV-1 that had been spiked at 10^6 $TCID_{50}$/mL. A reduction of 5.6 log_{10} was achieved with a one J/cm^2 UVA dose, and HIV-1 was reduced by greater than 6.4 log_{10} with a three J/cm^2 UVA dose (24).

In another study, S-59 and UVA produced a photochemical reaction that was able to inactivate cell-free HIV (>6.7 log_{10}), cell-associated HIV (6.6 log_{10}), DHBV (>6.8 log_{10}), and BVDV (>6.5 log_{10}) (25). Human CMV was also inactivated by S-59 and UVA. Freshly prepared platelet concentrates were suspended in plasma diluted with a synthetic platelet additive. CMV was inactivated by greater than four log_{10} after treatment with 1.5 μM of S-59 and one J/cm^2 of UVA illumination (26).

In another study, it was found that *4′-aminomethyl-4,5′,8-trimethylpsoralen (AMT) and UVA* can inactivate VSV and SIN (27). AMT with UVA treatment was shown to inactivate more than five log_{10} of model viruses and HIV. Although platelet in vitro properties were preserved, AMT is mutagenic (28,29). One article describes a method that can be used for determining residual AMT mutagenicity after platelet treatment with AMT and UVA (30).

AMT and UVA treatment of both free HIV cell-associated HIV in platelet suspensions was effective in inactivating four to five log_{10} of cell-free HIV. After treatment with AMT and UVA, no HIV replication was detectable in the infected cells. Furthermore, stable, integrated sequences of the HIV proviral *env* gene could no longer be amplified by PCR (31). Another study showed that three forms of HIV (cell-free, actively replicating, and latent) could be inactivated completely ($\geq 10^5$ infectious units) by AMT with rutin and UVA light (360–370 nm). The cells were suspended in plasma rather than a synthetic medium (32).

Cell-associated VSV in platelets was completely inactivated by 50 μg/mL of AMT with an irradiation time of 90 minutes. The presence of a quencher (such as the flavonoid rutin) is required to prevent oxygen-mediated damage to the platelets (33). Cell-free VSV in platelet concentrates was completely inactivated by AMT and UV using mannitol as a quencher to prevent platelet damage. The presence of mannitol did not interfere with VSV inactivation. Other quenchers that were evaluated include quercetin, glutathione, and SOD (34).

Inactivation of VSV and PRV by AMT was found to be dependent on plasma levels. A level of 14.5% and a concentration of 40 μg/mL of AMT were found to be optimal. A log_{10} reduction of about five was achieved for both VSV and PRV (35). AMT (25 μg/mL) and UVA irradiation for two hours provided a $\geq$5.4 log_{10} reduction of VSV in platelet concentrates.

Treatment with 300 μg/mL of *8-methoxypsoralen (8-MOP) and UVA* also inactivated VSV (six hours caused six log_{10} reduction). AMT, however, caused less aggregation of the platelets. (27). The psoralen 8-MOP was evaluated in the 1980s, but the photochemical treatment was not sufficiently rapid (36).

Both high-titer DHBV-infected sera and intracellular DHBV in platelet concentrates were inactivated completely by 8-MOP and UVA light. Inactivation was dependent on UVA dose but independent of 8-MOP concentrations of 100 to 300 μg /mL. A PCR-enhanced DHBV culture system was found to be useful in optimizing photochemical decontamination protocols (37).

Using PCR detection and the DHBV as a model for human HBV, a five to six log_{10} virus kill was possible. DHBV inactivation was dependent on UVA dose, plasma content, and the solubility of the 8-MOP (38). Using 8-MOP (300 μg/mL) and UVA treatment (17 mW/cm^2)

Table 3. Viral inactivation methods published since 1990 for viruses found in platelets

Viruses	Method	References
VSV, SIN, HIV	Aluminum phthalocyanine tetrasulfonates	14
HSV, VSV, PRV	Merocyanine 540	30,42
VSV	Phenothiazine dyes	29,41
HIV, CMV, SIV	Porphyrin derivatives and sapphyrin	20
HIV	Psoralens, S-59	25,26
DHBV, BVDV	Psoralens, S-59	26
CMV	Psoralens, S-59	27
HIV	Psoralens, 8-MOP	40
DHBV	Psoralens, 8-MOP	38,39
VSV, SIN	Psoralens, AMT	28
VSV, PRV	Psoralens, AMT	36
HIV	Psoralens, AMT	29,30,32,33
VSV	Psoralens, AMT	34,35
VSV	Psoralens, AMT, 8-MOP	28
Polio	Pulsed laser–UVB radiation	42

for 60 minutes inactivated cell-associated HIV-1 in platelet concentrates. A $\log_{10}$ reduction of six was obtained, and no virus was detectable. Adducts of 8-MOP–DNA were formed, but only when UVA was present (39).

Phenothiazine dyes. An investigation of VSV inactivation by photodynamic treatment with five different phenothiazine dyes (methylene blue, azure B, azure C, thionine, and toluidine blue) found that only thionine dye exceeded methylene blue virus inactivation efficiency (40). Methylene blue was capable of inactivating free virus while maintaining platelet integrity, but it was unable to inactivate intracellular viruses (28).

Porphyrin derivatives and sapphyrin. HIV-1, CMV, and SIV in both culture medium and blood were inactivated by several photosensitive dyes. Although these dyes caused no damage to RBCs (as mentioned earlier), platelets were modified over time. The most effective photoactive dyes were dihematoporphyrin ether (DHE), hematoporphyrin derivative (HPD), benzoporphyrin derivatives (BPD), and an alkyl-substituted sapphyrin (20).

Merocyanine 540 transfers energy to molecular (singlet) oxygen when it is exposed to visible light (450–600 nm). The oxygen then reacts with lipids and proteins and even with nucleic acids. HSV was sensitive to this photoinactivation method, but 5% albumin added to protect the cells interfered with the inactivation (41). Light and merocyanine 540 inactivated VSV and PRV by six $\log_{10}$ in the absence of added protein. But when 16% plasma was added, only 1.3 $\log_{10}$ of inactivation was obtained. The technique was judged unsuitable for platelets (29).

Pulsed laser–UVB radiation. At UVB doses of 10.5–21.5 J/cm^2, four to six $\log_{10}$ of polio were inactivated, and the platelets tolerated the treatment relatively well. At higher radiation intensities, however, the platelets were damaged (41).

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Part 3b, Plasma and Plasma Products
(Treatments Other than Heat or Solvent/Detergent)

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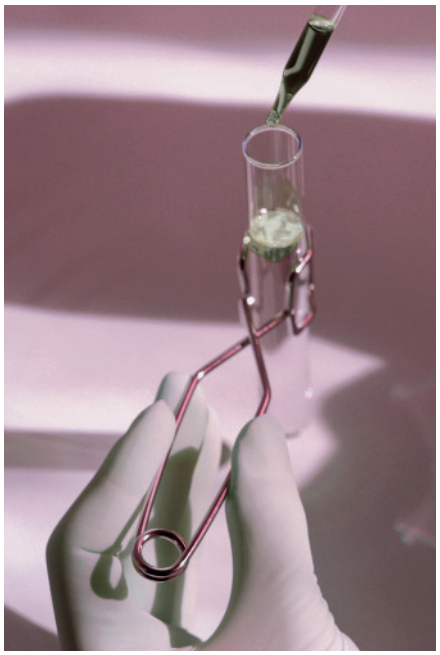

Gail Sofer

Viruses challenge biopharmaceutical manufacturers, and different viruses require different inactivation methods. This article series summarizes viral inactivation methods published during the last decade of the 20th century and into the year 2001. Part 1 discussed inactivation of viruses in skin, bone, and cells (1). Part 2 discussed red blood cells and platelets (2). Part 3, on plasma and plasma products, was divided into two separate articles: Part 3a on heat and solvent/detergent (S/D) inactivation methods (3), and this (part 3b) includes treatments other than heat and S/D.

Methylene Blue Phototreatment

Methylene blue phototreatment (fluorescent light for one hour at 60,000 lux) has been shown to inactivate cell-free, lipid-enveloped viruses in frozen plasma. It has also been shown to inactivate some nonenveloped viruses (such as ADV). Cell-free HIV in frozen plasma was inactivated by >6.7 $\log_{10}$. Filtration was used to remove cell-associated HIV infectivity (4).

Cell-associated viruses are first removed by freezing and thawing or by filtration. Dye is added first so that it associates with the viral membrane components or the viral nucleic acid, then fluorescent light at 45,000 lux is used to activate the dye. When exposed to light in the presence of oxygen, the virus nucleic acid or membrane components can be damaged by the singlet oxygen created (5). No virus inactivation was observed for EMC, polio, HAV, or PPV.

Methylene blue (1 μM) photodynamically inactivated viruses in fresh, frozen plasma by illuminating the single units of plasma in their plastic containers with fluorescent tubes. Table 1 shows these data and the specific illumination times required to achieve viral inactivation (5,6).

HIV-1 was spiked into fresh, frozen plasma and illuminated with visible light in the presence of 1 μM of methylene blue. After five minutes, the infective titer was reduced by 4.3 $\log_{10}$, and after 10 minutes, it was reduced below the limit of detection. Methylene blue without illumination, and illumination alone, both had minimal effect on HIV-1 inactivation (7).

HCV and HIV were spiked into fresh plasma, which was then treated with 1 μM of methylene blue. After one hour in the dark at 4°C, fluorescent lighting was applied. The effects of the treatment on the RNA of HCV and HIV-1 were studied by reverse transcriptase polymerase chain reaction (RT-PCR) analysis. A few minutes of illumination in the presence of methylene blue strongly reduced the detection of viral RNA. However for HIV, the effect of photosensitization on PCR signals was much smaller than on infectivity. The same was found to be true for HCV, but HCV was slightly more sensitive (8).

The inactivation of HSV-1 and SHV-1 by methylene blue and light was suppressed by the addition of plasma and by the presence of a singlet oxygen quencher (imidazole). The data indicated that singlet molecular oxygen is involved in virus inactivation by methylene blue and light (9). The target structures for HIV-1 inactivation by methylene blue and light are the envelope and core proteins and the inner core structures (10).

Methylene blue and light inactivation of enveloped viruses spiked into human plasma is time-dependent. SFV was inactivated in five to 10 minutes, but VSV took one hour for complete inactivation. HIV infectivity was removed within 10 minutes. DHBV could be inactivated, but required high doses of light. A methylene blue concentration of 0.3 μM was sufficient to reduce SFV infectivity below the detection limit in less than 15 minutes. For VSV inactivation, a concentration of 0.8–1.0 μM methylene blue was necessary with 45–60 minutes of illumination. A minimal light strength of 45,000 lux by fluorescent light was recommended for inactivation of viruses in fresh plasma. The light strength required is dependent on the light source. Most of the nonenveloped viruses, however, were resistant to methylene blue and light treatment. SV-40, calicivirus, and ADV were somewhat susceptible to inactivation. EMC, MEV, HAV, and PPV were not reduced after one hour. Polio was not reduced after two hours (11).

Inactivation of VSV in plasma by methylene blue with and without illumination was studied. In the absence of dye, illumination for four hours led to only marginal inactivation. Low concentrations of methylene blue in the dark also had minimal effect. More than six $\log_{10}$ VSV were inactivated with 1 μM of methylene blue in combination with light. Influenza virus required a five- to 10-fold higher methylene blue concentration than did VSV or HSV (12).

The efficacy of virus inactivation by methylene blue depends on light irradiance, duration of irradiation, bag volume, and methylene blue concentration (13). Fresh, frozen plasma treated with methylene blue was taken off the market in Germany after it was found that incomplete removal of methylene blue could result in genetic toxicity. However, methylene blue is still used in several applications (14).

Three bags of plasma can be illuminated together after adding a dry methylene blue pill that results (after it is dissolved) in a concentration of 1 μM (15).

Chlorin-Type Photosensitizer

The ability of a chlorin-type molecule to photoinactivate a series of HIV-1 strains in buffer, plasma, and whole blood was investigated. Complete inactivation of HIV-1 in plasma was obtained with a reasonable light dose (661 nm) (16).

3,3′-(1,4-Naphthylidene) Dipropionate

Inactivation of HSV-1 and SHV-1 by singlet oxygen produced by 3,3′-(1,4-naphthylidene) dipropionate ($NDPO_2$) provided good inactivation in phosphate-buffered saline (PBS).

The presence of 80% v/v human plasma, however, diminished the amount of virus killed, and a higher concentration of $NDPO_2$ was required. Using 30 mM $NDPO_2$, inactivation of three $\log_{10}$ of SHV-1 was obtained (9).

Psoralens

Photochemical inactivation of viruses in plasma and plasma fractions by 8-methoxypsoralen (8-MOP) and UVA reduced HCV by four $\log_{10}$ and HBV in factor 8 (FVIII) concentrate by 4.5 $\log_{10}$. BTV in plasma was inactivated by aminomethyl trimethylpsoralen (AMT). VSV and FeLV in FVIII concentrate were inactivated by 8-MOP (17).

Beta-Propiolactone

Cryosupernatant produced during plasma fractionation can be treated with 0.25% β-propiolactone at 4°C for five hours at pH 7.2 to inactivate both enveloped and nonenveloped viruses during the production of immunoglobulin, albumin, and factor 9 (FIX) (18). FVIII, however, is unstable in the presence of β-propiolactone. For immunoglobulin G (IgG) solutions, 0.1% β-propiolactone, at pH 8.1 and 20–22°C for eight hours, provides inactivation factors about 10% higher than those found in plasma. FCV inactivation was 7.2 $\log_{10}$ in plasma and 8.12 $\log_{10}$ in IgG (19).

The capability of β-propiolactone (0.25% at 4°C) to inactivate viruses was affected differently by the presence of cryoprecipitate-poor (cryo-poor) plasma and IgG. In the presence of IgG or cryo-poor plasma, SFV, VSV, BVDV, and MEV were effectively inactivated. In IgG, SIV, BHV-1, SHV-1, and FCV were also sufficiently inactivated. In plasma, however, SIV, HIV-2, BHV-1, SV-40, FCV, and PPV were not sufficiently inactivated by this treatment. Viral reduction factors were:

IgG: 4–5.5 $\log_{10}$ for VSV, SFV, BVDV, MEV, FCV, PPV, and SV40

2–4 $\log_{10}$ for SHV-1, BHV-1, HIV-2, and SIV

Plasma: 3–5 $\log_{10}$ for VSV, SFV, BVDV, SHV-1, and MEV

0–3 $\log_{10}$ for HIV-1, SIV, BHV-1, FCV, PPV, and SV40.

There was also variability in the inactivation kinetics for the various viruses (20).

In the presence of cryo-poor plasma, β-propiolactone (0.25% at 5°C) provided insufficient inactivation of HIV-1. The reduction was only 1.0 $\log_{10}$ after one hour and 1.8 $\log_{10}$ after five hours (21).

β-propiolactone was validated as a viral inactivation method in the production of two intravenous immunoglobulin (IVIG) preparations. A β-propiolactone concentration of 0.1% (w/w) was used for virus inactivation in a 4% immunoglobulin solution at 22°C. In both immunoglobulins, HIV-1, PRV, VSV, BVDV, and EAV were reduced below the detection limit after 480 minutes. SV40 showed a significant reduction after 480 minutes. In 300 minutes, PPV was reduced more than five $\log_{10}$ and BHV-1 by more than 4.6 $\log_{10}$. SIV was reduced by more than 2.5 $\log_{10}$ in 15 minutes, SFV by more than 5.4 $\log_{10}$ in 120 minutes, calicivirus by more than 6.3 $\log_{10}$ in 90 minutes, and MEV by more than 5.1 $\log_{10}$ in 45 minutes (18).

β-propiolactone with UV light was shown to inactivate 7.0 $\log_{10}$ of HBV, ⩾4.5 $\log_{10}$ of HCV, and ⩾6.0 $\log_{10}$ of HIV (22). Table 2 lists the references for the various viruses in plasma and plasma products inactivated with β-propiolactone.

Table 1. Photodynamic inactivation of various viruses in fresh plasma using methylene blue (1 μM) with different times of illumination to achieve inactivation (6)

Virus	$\log_{10}$ Inactivation[a]	$\log_{10}$ Inactivation[b]	Illumination Time[b,c]
ADV	4.00	4	120
BHV	⩾8.11	>4.86	30
BVDV	⩾5.63, ⩾6.41		
Calici	>3.90	>3.9	5
Classical swine fever	⩾3.20		
EMC	0	0	60
HAV	0		
HIV-1	⩾6.32	>4.00	10
HIV-2	⩾3.81	>1.76	10
HSV	⩾5.50	>5.5	60
Influenza	5.10	5.1	60
Polio	0	0	120
PPV	0	0	60
Reo-3	3.80		
Semliki forest	⩾8.77	>7.00	10
SIN	⩾9.73	>9.73	5
SIV	⩾6.26	>3.29	15
Suid herpes type 1	4.43	>4.52	60
SV-40	>4.00	>4	30
VSV	⩾4.89	>4.89	60
West Nile	⩾4.39		

[a]Reference 5 (Mohr 2000) [b]Reference 6 (Mohr 1993) [c]In minutes.

Table 2. References that discussed virus inactivation performed in plasma and plasma products using β-propiolactone

Viruses	Reference
FCV	19
BHV, BVDV, FCV, HIV-2, MEV, PPV, SFV, SHV, SIV, SV-40, VSV	20
HIV-1	21
BHV, BVDV, calicivirus, EAV, HIV-1, MEV, PRV, PPV,SFV, SIV, SV-40, VSV	18
HBV[a], HCV[a], HIV[a]	22

[a]Plus UV

S-Sulfonation

Oxidative sulfonation of a 7s immunoglobulin provided the following $\log_{10}$ reduction values: BVDV >5.0, TBEV >7.3, YFV >6.3, HIV-1 >4.0, HIV-2 >5.6, HSV-1 >4.6, and polio-3 >4.4 (23). S-sulfonation was able to inactivate HIV-1 (24).

Hydrostatic Pressure

Hydrostatic pressure cycling was used to inactivate virus in human plasma. Lambda phage (λ phage) was used as a model virus. When performed at near 0°C, λ phage titers were reduced by approximately six $\log_{10}$ after 10 to 20 minutes (25).

Iodine

Iodine/Sephadex (Amersham Biosciences, www.apbiotech.com) has been shown to deliver iodine to IVIG in a slow, controlled way that inactivates >4.2 $\log_{10}$ PPV, >4.7 $\log_{10}$ BVDV, >4.2 $\log_{10}$ PRV, and >8.1 $\log_{10}$ EMC (26).

Viruses spiked into an antithrombin III complex containing up to 0.1% human albumin were inactivated by liquid iodine. Log_{10} reduction values were greater than six for SIN, EMC, and VSV, greater than four for PRV, and greater than three for HIV. With the exception of SIN, inactivation was complete (27).

Table 3. The effect of variables on pH and pepsin inactivation tests on IgG

pH	Pepsin	Other Variables	Temperature °C	VSV	BVDV	SFV	PRV	HIV	HSV	CMV	HCV
4.0	+		37		+, 5 min	+, 5 min	+, 30 min	+, 30 min			
4.0	+		4		slow	slow	slow	slow			
7.0	−		37 or 4		N[a]	N[a]	N[a]	N[a]			
4.0	+	sucrose	37			N[a]	slow				
4.0	+	NaCl	37			slow	N[a]				
4.0	+	IgG 1%	37			fast					
4.0	+	IgG 10%	37			slow					
4.0	+	IgG 6%	37	fast							
4.0	+	IgG 16%		slow							
4.0	+		37	5.7		+		+	+	+	
4.0	−		37	3.9		+		+	+	+	
4.0	+		37					+			
4.0	−		37					+			
4.25	−		21		+						+

[a]Negligible or no effect

Cross-linked starch–iodine was shown to be an effective inactivation method for both lipid-enveloped and nonenveloped viruses in plasma. An iodine concentration of 1.05 mg/mL and a 60-minute incubation time inactivated more than nine $\log_{10}$ VSV and more than seven $\log_{10}$ of EMC (28).

VSV spiked into cryo-poor plasma was treated with cross-linked povidone iodine at concentrations from four to 10 mg/mL for up to 120 minutes at 4°C. At 24°C, the 10 mg/mL treatment inactivated more than seven $\log_{10}$ of VSV within five minutes (29).

A polyvinylpolypyrrolidone (PVPP) and iodine complex in a depth filter was used for viral inactivation of VSV in 2% IgG. The $\log_{10}$ reduction was >7.3. Filtration was at room temperature, at pH 7.4, with a filtration speed of about 500 Lm^2/h. Inactivation of several different species of parvovirus was between four and six $\log_{10}$ in 2–5% IgG, FIX solution (0.44 mg/mL), or buffered saline, pH 7.0. Reo-3 was inactivated by 4.9 $\log_{10}$ (the available starting titer) in a 2% IgG solution. For parvovirus and Reo-3, the filtration speed was 100–500 Lm^2/h and the temperature was either 40°C or room temperature (30).

Caprylate

Caprylic acid (99% octanoic acid) can inactivate lipid-enveloped viruses in plasma products. In albumin, VSV was inactivated in 60 minutes or less (31). Caprylate has also been used with amino acids and heat as a virus elimination method in the production of FVIII. At a recent conference, Bayer Corporation (www.bayer.com) discussed the use of caprylate as a very robust inactivation method for an IVIG (32). Twelve mM of caprylate (0.2% at 25°C, pH 5.1) provided a $\log_{10}$ reduction of ≥4.4 for BVDV, which has been described as the most caprylate-resistant enveloped virus associated with the plasma fractionation processes. Caprylate at a 40 mM concentration, at pH 5.4, 40°C, also inactivated BVDV in an albumin solution. The presenter also found that the rate of inactivation of BVDV by 19 mM caprylate in a fraction II + III solution was 20 to 60-fold faster than inactivation by solvent/detergent (S/D) in filtrate III (32).

UVC Irradiation

A fibrinogen solution (containing rutin to protect the fibrinogen) was irradiated with UVC (254 nm, 0.1 J/cm^2). Nonlipid viruses inactivated were PPV (≥5.5 $\log_{10}$), EMC (≥6.5 $\log_{10}$), and HAV (≥6.5 $\log_{10}$). Inactivated lipid-enveloped viruses included HIV (≥5.7$\log_{10}$) and VSV (≥5.7 $\log_{10}$) (33).

UVC irradiation was also used for virus inactivation in plasma and in a FVIII concentrate. UVC light targets nucleic acids and was shown to inactivate EMC, HAV, VSV, and PPV. In the FVIII concentrate, all four viruses were inactivated to undetectable levels. Log_{10} reductions were ≥4.7 for EMC, ≥6.3 for HAV, ≥4.6 for VSV, and ≥4.8 for PPV. In plasma, all four viruses were completely killed (34).

UVC was effective in inactivating polio-2, SFV, HSV, and vaccinia in albumin and IVIG preparations. UVC was found to be more effective than UVB (280–320 nm). Virus inactivation occurred at or before five minutes and provided $\log_{10}$ reductions of 3.4 for SFV, ≥6.6 for vaccinia, ≥6.4 for polio, and ≥5.4 for HSV (35).

UVC light was used to inactivate parvovirus B19 in coagulation factor concentrates. B19 was inactivated by three $\log_{10}$ at 750 J/cm^2 and was undetectable after 1,000 or 2,000 J/cm^2. Rutin or catechins were required to maintain biological activity. With epigallocatechin gallate, FVIII activity was retained almost 100% and B19 was decreased to undetectable levels (>3.9 $\log_{10}$) (36).

Pulsed Light

Pulsed power and broad spectrum light has been shown to cause dimer formation and DNA and RNA strand breakage at low energy levels that do not appear to destroy proteins. The lights tested were UVA, UVB, UVC (200–400 nm), visible (400–780 nm), and infrared (780 to >100 nm). The optical properties of the solution are important. Alpha-1 (α-1) proteinase inhibitor, IgG, and monoclonal antibodies have been tested. Over 26 different viruses have been shown to be inactivated by this technology (37).

Gamma Irradiation

Gamma irradiation in the presence of fibrinogen, FVIII, and α-1 proteinase inhibitor at doses of 23, 28, and 30 kGy, respectively, was able to inactivate four $\log_{10}$ of PPV. BVDV was completely inactivated by 20–30 kGy in these products, but gamma irradiation was less effective in inactivating viruses in freeze–dried immunoglobulin (26).

Inactivation of HIV by gamma irradiation was studied in both frozen and liquid plasma. The dose required to inactivate five to six $\log_{10}$ of HIV was 50–100 kGy at −80°C and 25 kGy at 15°C. At those doses, however, less than 85% of the biological activity of plasma products was retained. The tolerable dose for plasma irradiated at −80°C should not exceed 14 kGy. For lyophilized FVIII concentrate, the dose should not be more than two and four kGy at 15°C and −80°C, respectively. Hiemstra et al. concluded that at those doses, HIV is not

adequately inactivated and that gamma irradiation should not be used for sterilization of plasma and plasma products (38).

Pepsin and Low pH

Virus inactivation of IgG preparations by treating them with pH 4 and pepsin has been used as part of the manufacturing process for IgG for intravenous use. Factors found to influence the inactivation kinetics include temperature and content of the IgG solution. At 37°C, inactivation of BVDV and SFV was rapid and complete to the limit of detection within five minutes of incubation. Complete inactivation of PRV and HIV, however, was slower and required about 30 minutes. Log_{10} reduction factors were >4.4, >6.8, >5.6, and >5.0 for BVDV, SFV, PRV, and HIV, respectively.

Temperature, sucrose, NaCl, and IgG concentration. A marked decrease in inactivation rates for all viruses was found at temperatures below 37°C, with the lowest rates at 4°C. At neutral pH, where no pepsin activity was detectable, virus inactivation was negligible at both 37°C and 4°C. Increasing the sucrose concentration in the IgG solutions inhibited the rate of inactivation of PRV but not the inactivation rate of SFV. Increasing the NaCl concentration decreased the rate of inactivation of SFV, but the effects on PRV inactivation were inconsistent. Increases in IgG concentrations had no clear effect on PRV inactivation, but decreased the rate of SFV inactivation (39).

The effects of time, temperature, pH, and stabilizers (sucrose and NaCl) on inactivation of lipid-enveloped model viruses, SFV and VSV, in the IVIG production was investigated. Lowering the pH, raising the temperature, and increasing the incubation time improved inactivation. Small changes in pH and stabilizer concentrations failed to influence the results. Protein concentration had an effect. VSV was inactivated several logs faster in IVIG (6% protein) than in immunoglobulin intramuscular (IGIM), which was 16% protein. Complete inactivation required incubation of 20 hours or more. Freeze–drying in the presence of ethanol or storage in a liquid state at pH 7 only partially inactivated SFV and VSV (40).

After fractionation of immunoglobulins using the Kistler–Nitschmann method, SFV, VSV, CMV, HSV-1, and HIV were spiked into the immunoglobulin preparations and treated for 16 hours at 37°C. The pH was adjusted to either seven or four. All viruses, except VSV, were totally inactivated after incubation at pH 4 with or without pepsin. Incubation in IgG solutions at pH 7 resulted in total inactivation of HSV and CMV, which may be because neutralizing antibodies were present. Log_{10} reduction factors at pH 4 were greater than six for SFV, HSV-1, CMV, and HIV. For VSV, log_{10} reductions were 3.9 without pepsin and 5.7 with pepsin. Studies on SFV showed that temperature alone had little effect, but the amount of virus spike did seem to affect the survival of virus. At higher virus concentrations, the viral particles appeared to exhibit a self-protecting effect that could have been a result of aggregation (41).

IgG solutions were spiked with HIV at more than six log_{10}. At 37°C and pH 4, HIV was totally inactivated in two hours, with or without pepsin (42). Table 3 highlights the effect of variables on inactivation using pH and pepsin in IgG.

Low pH

Inactivation of HCV by low pH was investigated in an IVIG final solution. At pH 4.25 for 21 days at 21°C, the model virus (BVDV) titer decreased 10,000-fold. After seven days, PCR assays showed a 10-fold decrease, and that decrease remained constant. Complete inactivation was also found for 1,000 chimpanzee infectious doses of HCV (43). Viral inactivation by low pH will be discussed further in a future article of this series, which will summarize inactivation methods in biotechnology products.

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