

Pathogen Inactivation of Blood Components: Current Status and Introduction of an Approach Using Riboflavin as a Photosensitizer

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Abstract

Riboflavin is a naturally occurring compound and an essential human nutrient. Studies in the 1960s and 70s showed that it could be effective, when exposed to visible or UV light, in inactivating viruses and bacteria [1]. This suggested to us that it could act as a photosensitizer useful in the inactivation of pathogens found in blood products, because of its nucleic acid specificity and its limited tendency toward indiscriminate oxidation. The riboflavin molecule is a planar, conjugated ring structure with a sugar side chain that confers water solubility. The planar portion is capable of intercalating between the bases of DNA or RNA. Light activated riboflavin oxidizes guanine in nucleic acids, preventing replication of the pathogen's genome [2]. Gambro BCT is developing processes using riboflavin and light to inactivate pathogens in plasma, platelet, and red cell products. We call these Pathogen Eradication Technology (PET) processes. Riboflavin is non-toxic; it must be present in the body for good health. The photo-byproducts formed in the PET processes are lumichrome and protein adducts. The photodegradation of riboflavin in the body is clearly shown by the decrease in its concentration in neonates who are treated with intense visible light to break down circulating bilirubin, which their immature livers cannot yet handle [3]. A definitive lookback study showed no difference in cancer rates between the 55,000 children receiving this therapy in Denmark from 1977 through 1989 and nonirradiated controls [4]. Gambro BCT is developing specific riboflavin-based PET processes for platelet concentrates, fresh frozen plasma, and packed red blood cells. In each, the process is being optimized to achieve high levels of inactivation of specific pathogens, while maintaining acceptable levels of product quality and activity. Extra- and intracellular HIV, BVDV (a model for HCV), and pseudorabies virus (a herpes virus) have been used to guide process development and validation. We have demonstrated 4 to 7 log₁₀ reductions in the titers of these viruses, when they are spiked into blood products and irradiated in the presence of riboflavin. Porcine parvovirus, a tight-capsid, nonenveloped virus is more resistant, a finding in all experimental inactivation approaches. A range of bacteria implicated in platelet and red cell transfusion injuries and deaths, including *S. aureus*, *E. coli*, *K. pneumoniae*, and *Y. enterocolitica*, are being used to validate antibacterial efficacy. The PET platelet process involves the addition of riboflavin to platelets in plasma, illumination of the product, storage of the product and transfusion without further manipulation. The lack of toxicity of the treatment byproducts permits this ease of use. Quality of the platelets throughout storage has been assessed by pH, PO₂, lactate, hypotonic shock response, morphology, glucose, and GMP-140 expression. In vitro function is well maintained. The levels seen are within the range of those reported in commonly transfused products. Radiolabeled transfusion studies of treated platelets have been carried out in primates to determine a preliminary measure of their in-vivo circulation. The in vivo recoveries and survivals of treated and control platelets did not differ. This work suggests that an endogenous photosensitizer, riboflavin, which has an extremely good safety profile, can inactivate high levels of a broad range of viruses and bacteria in platelet concentrates, fresh frozen plasma, and in red blood cells, preserving the activity and functionality of the components. Planned animal and clinical studies are expected to solidify this suggestion into a

well-characterized process which can be safely and readily applied to reduce the risks of transfusion transmitted disease.

1. Tsugita A, et al. Photosensitized inactivation of nucleic acids in the presence of riboflavin. *Biochim Biophys Acta*. 1965;103:360-363.
2. Douli T, Cadet J. Modification of DNA bases by photosensitized one-electron oxidation. *Int J Radiat Biol*. 1999;75:571-581.
3. Sisson TRC. Photodegradation of riboflavin in neonates. *Fed Proc*. 1987;46:1883-1885.
4. Olsen JH, et al. Childhood leukemia following phototherapy for neonatal hyperbilirubinemia (Denmark). *Cancer Causes and Control*. 1996;7:411-414.

Gambro BCT, Inc. is developing a novel approach to reducing the risk of transfusion transmitted diseases using a common vitamin, riboflavin, and light. This approach to enhancing blood safety could be a significant benefit not only in industrialized countries but also in areas where unscreened blood and high endemic viral incidence today result in significant risk of transfusion transmitted disease.

1. Background

The appearance of transfusion-transmitted Acquired Immune Deficiency Syndrome (AIDS) in 1980 and its devastating effect on thousands of transfused patients, in particular hemophiliacs who were treated with products made from large pools of donor plasma, changed attitudes toward blood safety forever. Enhanced donor screening based on epidemiological evidence of the source of the infection was implemented. A series of increasingly sensitive new tests for the presence of HIV were added in many countries. Tests for hepatitis C virus (HCV) and human T cell lymphotropic virus (HTLV) soon followed. Viral safety for blood products became a mantra in modern medicine.

Recently introduced tests amplifying and detecting specific viral nucleic acid sequences (NAT) have reduced the risk of infective doses of HIV and HCV in transfused blood even further. The table below is an estimation of these based on published data [1-4].

Virus	Risk of infective dose per million donations
HIV	0.3 to 1 per million
HCV	1 to 3 per million
HBV	5 to 10 per million
HTLV	1 to 2 per million

While these are very low risks, each donation may result in multiple products, and patients often receive multiple transfusions. In the areas noted, using state-of-the-art nucleic acid testing (NAT) and serological testing, 500 to 1000 patients per year are expected to receive infective doses of one of the viruses noted in the table.

In addition to viruses, bacteria and parasites represent infective risks in blood products. Bacteria may be

present as infections in the donor or, more frequently, enter the blood products by way of the venipuncture used to collect them. Products contaminated in this way usually have low numbers of bacteria levels that can be readily handled by the body's immune systems or by antibiotics used prophylactically in transfusion recipients who have seriously impaired immune responses. However, in platelet products stored up to 5 days at room temperature, the inocula can multiply to high levels and can cause serious morbidity or death if transfused [5]. Use of single donor platelet transfusions versus concentrates prepared from pooled platelets from many donations reduces the risk because they involve fewer venipunctures [6]. Data from a recent large retrospective study [6] can be used to estimate the risk of serious outcomes due to bacterially contaminated platelet products. These estimates are shown in the following table:

Platelet product	Risk of septic reaction per million transfusions	Risk of septic death per million transfusions
Pooled platelets from whole blood	400	60
Single donor apheresis platelets	75	15

Risks today for serious septic outcomes from red blood cell transfusion are lower than those for platelet transfusion, because of the cold storage temperatures for packed red cells. Some bacteria, however, can multiply at low temperature and serious outcomes, including fatalities, have resulted. The estimated risks, in the absence of bacterial screening, for serious outcomes are 6/million with death resulting in nearly 20% of these cases [7].

The incidence of parasitic infections from transfusion, such as malaria and Chagas' disease, varies with geographical region. In endemic areas they represent a serious risk. In most countries, the transfusion risks are handled by questioning the donor about recent travel and deferring those who have traveled in endemic areas. [1].

2. Public Expectations Regarding Blood Safety

Social and political pressure is being applied to the blood industry to achieve a zero risk blood supply. Ignoring the statistical naivet of this goal, the directive is

clear: use all means currently available and develop new methods for improving transfusion safety. One aspect of transfusion risk, understood by the public and by medical professionals, is that of a new agent entering the blood supply, which causes serious morbidity or mortality and goes undetected by the testing in place; essentially a reenactment of the transfusion transmitted AIDS epidemic. Fear of the unidentified virus, along with the understanding that we have reached a point of diminishing returns in direct testing for the presence of identified, dangerous viruses, provides the impetus for the development of technologies for the active elimination of viruses and other pathogens from blood components through their inactivation or removal. In most cases inactivation of pathogens present in blood products is seen as the more feasible approach for very high levels of risk reduction.

3. Expectations of Pathogen Inactivation of Blood Products

The most desirable pathogen inactivation process might have the following characteristics:

- Reduce to negligible levels the incidence of viral, bacterial and parasitic transmission by blood components
- Have robust and broad enough effectiveness to prevent a transfusion related epidemic of a dangerous new pathogen
- Be easily implemented into the blood banking system
- Introduce no new risks to transfused patients or to the staff carrying out the process, i.e. negligible toxicity
- Retain the full clinical effectiveness of the transfusion product
- Have low or reasonable economic costs
- Offers a simple integration into blood banks operations

No process will perfectly meet all these desires. Different approaches will trade some off against others. A number of pathogen inactivation technologies are already in use for fresh frozen plasma (e.g. solvent detergent and methylene blue). Reduction of blood component quality, gaps in the spectrum of pathogens covered, and the lack of applicability to red blood cells and platelets are the key drawbacks to these approaches.

4. Riboflavin Photosensitized Pathogen Inactivation

Gambro BCT is developing processes using riboflavin, or vitamin B₂, as a photosensitizer to inactivate pathogens in plasma, platelet, and red cell products. Japanese scientists demonstrated in the 1960s that riboflavin, when exposed to visible or UV light, could be used to inactivate the RNA containing tobacco mosaic virus [8]. The riboflavin molecule is a planar, conjugated ring structure with a sugar side chain that confers water solubility. The planar portion is capable of intercalating

between the bases pairs of DNA or RNA in a pathogen and absorbing light in the visible and near UV regions. Light activated, intercalated riboflavin oxidizes nucleic acids through electron transfer reactions [9] preventing replication of the pathogen's genome. Riboflavin, in the presence of light also produces reactive oxygen species, which, as with other photochemical approaches, cause less specific oxidative damage, contributing to the destruction of pathogens, but also potentially damaging the transfusion product. The fact that riboflavin is an endogenous chemical and that its metabolic products are the same as its photoproducts suggested that it should have no untoward effect on a transfusion recipient.

Risk of viral and bacterial transmission through blood products is today very low in many countries. Therefore, any new risks associated with a pathogen inactivation process should be even lower to justify using the process. The use of an endogenous, well-studied compound such as the vitamin riboflavin could provide a means to avoid the unknown risks of using newly synthesized compounds.

Riboflavin is non-toxic, as attested to by a very broad range of testing assessed prior to its designation as "Generally Recognized As Safe" by the USDA. It is important, however, to assess the toxicity not only of the starting material in a photosensitization process, but also of the products formed by its photochemical reactions. The only small molecular byproduct formed in the photolysis of riboflavin is lumichrome, which is also present in many foods. It is formed in the normal metabolic handling of ingested riboflavin [11]. We have also shown by Ames testing, mouse micronucleus testing, and chromosomal aberration testing that lumichrome, like riboflavin, is non-mutagenic and non-clastogenic.

Riboflavin photochemistry is known to occur *in vivo*. This is clearly shown by the decrease in riboflavin concentration in neonates, who are treated with intense visible light to break down circulating bilirubin, which their immature livers cannot yet handle. Riboflavin and bilirubin both absorb light in the visible region directly through the skin. Both are broken down to form photoproducts. The photolysis of circulating riboflavin during phototherapy has led to riboflavin supplementation in these neonates [13].

Because of riboflavin's known photoreactions with DNA, questions were raised about the potential for mutagenic or carcinogenic effects secondary to neonatal phototherapy [14]. A study, published in 1996, showed no difference in cancer rates between the 55,000 children receiving this phototherapy in Denmark from 1977 through 1989 and nonirradiated controls [15]. In addition, our internal testing clearly demonstrates the absence of genotoxicity of not only lumichrome but of the entire treated blood product. Additional toxicology testing, including pharmacokinetics, neoantigenicity, and acute and subchronic toxicity, is also negative. This testing confirms that photoproducts need not be removed from the treated blood components prior to storage and transfusion. This greatly simplifies the process.

We are developing specific riboflavin-based processes

for platelet concentrates, fresh frozen plasma, and packed red blood cells. Riboflavin acts as a photosensitizer in each process, but because of the differing characteristics of each component, for example, light transmission, sensitivity to ultraviolet light, etc. the optimal process will vary. A number of virus models, including extra- and intracellular HIV, have been used to guide process development and validation. A range of bacteria implicated in platelet and red cell transfusion injuries and deaths are being used to validate antibacterial efficacy.

5. Platelets

The platelet process involves the addition of riboflavin solution to platelets in plasma, illumination of the product with ultraviolet light, storage of the product and transfusion without further manipulation.

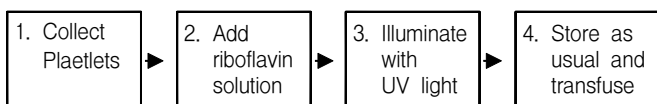


Figure 1. Schematic of the Riboflavin photoinactivation process for platelets.

Viral kill in platelets has been analyzed by spiking the concentrates with high concentrations of the test virus, determining the initial concentration, treating the riboflavin photoinactivation, and determining the viral concentration in the treated product. The viral reductions seen are shown in the following table:

Virus	Reduction Factor-Log
Pseudorabies	6.2
Porcine Parvo Virus	≥ 8.0
BVDV	5.75
Intracellular HIV	6.46

Quality of the platelets throughout storage has been assessed by pH, PO₂, lactate, hypotonic shock response, morphology, glucose, and GMP-140 expression. In vitro function is well maintained, the greatest differences from controls being in hypotonic shock response as seen in the following table.

	Control Day 5	Treated Day 5
pH	7.4±0.1	7.0±0.1
Lactate	6.2±1.1	12.2±1.4
HSR	79.6±7.4	46.4±6.6
Morphology	310±25	300±40
P-selectin	19.1±69.2	44.8±12.0

The levels seen, however, are within the range of those reported in commonly transfused products. Radio-labeled transfusion studies of treated platelets have been carried out in primates to determine their in-vivo circulation [16]. The in vivo recoveries and survivals of treated and control platelets are similar in this model as seen in the following figure.

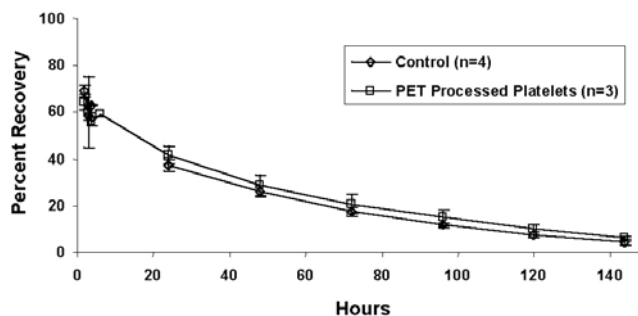


Figure 2. Autologous recovery of ¹¹¹In radiolabeled platelets treated by the Riboflavin photoinactivation process in Cynomolgus monkeys.

6. Plasma

The plasma process introduces riboflavin by a minimal dilution of the plasma, followed by illumination with UV light and freezing. The process gives greater than a 4 log reduction of many model viruses, including porcine and canine parvoviruses as shown in the figure below.

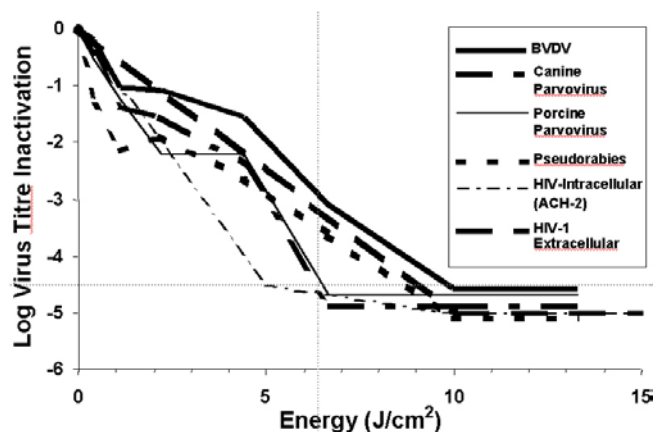


Figure 3. Inactivation of Viruses in FFP using riboflavin and UV Light.

These non-enveloped species are noted to be difficult to kill [17]. The thawed product may be transfused without further manipulation. The recovery of clotting factor activity is shown in the following table compared to Normal Reference Ranges.

Test	PET Treated Plasma	Normal Reference Range*
Fibrinogen (Mg/dl)	217±35	200-400
Factor 2 (IU/ml)	0.96±0.19	0.50-1.50
Factor 5 (IU/ml)	0.62±0.11	0.50-1.50
Factor 7 (IU/ml)	0.59±0.12	0.50-1.50
Factor 8 (IU/ml)	0.71±0.23	0.50-1.50
Factor 9 (IU/ml)	0.59±0.23	0.50-1.50
Factor 10 (IU/ml)	0.61±0.10	0.50-1.50
Factor 11 (IU/ml)	0.31±0.04	0.50-1.50

* Hemostasis and Thrombosis in the Clinical Laboratory (1988)
Edited by Corriveau DM and Fritsma GA, Page 116-119

7. Red Blood Cells

The riboflavin photoinactivation process has been successfully applied to red blood cells in preliminary experiments. The cells are washed, diluted in riboflavin solution and illuminated with visible light in a wavelength region where hemoglobin has a low absorbance. Good viral inactivation and subsequent storage of the cells for 42 days without excessive hemolysis were observed [18].

This work suggests that an endogenous photosensitizer, riboflavin, which has an extremely good safety profile, can inactivate high levels of a broad range of viruses and bacteria in platelet concentrates, fresh frozen plasma, and in red blood cells, while preserving the activity and functionality of the components. Planned animal and clinical studies are expected to solidify this suggestion into a well-characterized process, which can be safely and easily applied to reduce the risks of transfusion transmitted disease. If successfully brought to market, this process could greatly enhance public trust in the safety of the blood supply.

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