TABLE 3. Log_{10} reduction of bacteria and spirochetes in platelets

	INTERCEPT[71]	Mirasol[66]	Theraflex UV[65]
Gram Negative			
Escherichia coli	>6.4	>4.4	≥4.0
Serratia marcescens	>6.7	4.0	≥5.0
Klebsiella pneumoniae	>5.6		4.8
Pseudomonas aeruginosa	4.5	>4.5, >4.7*	≥4.9
Salmonella choleraesuis	>6.2		
Yersinia enterocolitica	>5.9		
Enterobacter cloacae	5.9		≥4.3
Gram Positive			
Staphylococcus epidermidis	>6.6	4.2	4.8
S. aureus	6.6	3.6	≥4.8
S. aureus MRSA Strain		4.8	
Streptococcus pyogenes	>6.8		
Listeria monocytogenes	>6.3		
Corynebacterium minutissimum	>6.3		
Bacillus cereus (includes spores)	3.6		
B. cereus (vegetative)	>5.5	1.9†	4.3
B. cereus (isolated from donated blood)		2.7	
Bifidobacterium adolescentis	>6.0		
Propionibacterium acnes	>6.2		4.5
Lactobacillus species	>6.4		
Clostridium perfringens (vegetative form)	>6.5		≥4.7
Spirochetes			
Treponema pallidum	≥6.8 to ≤7.0		
Borrelia burgdorferi	>6.8		

* ATCC 43088 and ATCC 27853, respectively.
† ATCC 7064.

TABLE 4. Log_{10} reduction of protozoa/rickettsia in platelets

	INTERCEPT	Mirasol
Plasmodium falciparum	≥6.0[72]	
Trypanosoma cruzi	>5.3[73,74]	6.0[75]
Leishmania mexicana	>5.0[76]	
Leishmania major, strain Jish	>4.3[76]	>5.0[77]
Babesia microti	>5.3[72]	
Orientia tsutsugamushi		>5.0[78]

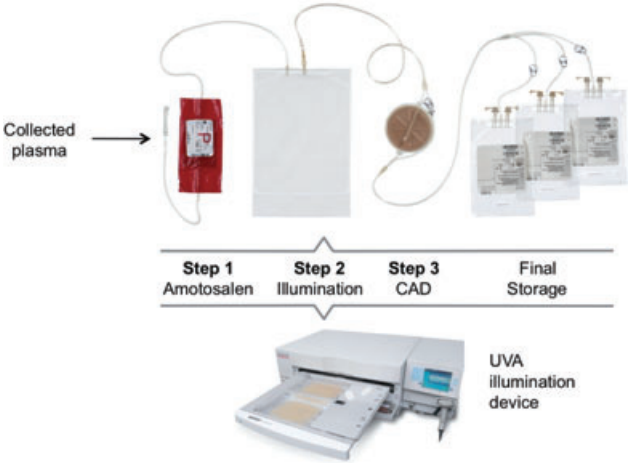

Fig. 5. Cerus Corporation INTERCEPT Blood System for plasma.[83]

their respective platelet PR system outlined above with some minor modifications. MacoPharma (Theraflex© MB-plasma) has developed a photochemical process that incorporates methylene blue (MB) and visible light. None is approved for clinical use in the US or Canada. These processes are shown in Figs. 5 through 7, respectively.

Octapharma (Lachen, Switzerland; http://www.octapharma.com) developed a PR process for pooled plasma units intended for large-scale manufacturing of what is commonly known as solvent/detergent (S/D) plasma (Octaplas).[79,80] A slightly different S/D plasma product manufactured by Vitex was licensed in the US but is no longer marketed.[79,81,82]

Cerus Corporation INTERCEPT Blood System for plasma

The INTERCEPT system for plasma is similar to their platelet system. After addition of amotosalen to the plasma and illumination, the plasma flows through a Compound Adsorption Device (CAD) to remove unreacted amotosalen and photoproducts. The process is shown in Fig. 5.

CaridianBCT Biotechnologies Mirasol plasma system

CaridianBCT Biotechnologies plasma system requires a transfer of the plasma into a final freezing/storage container after illumination. This process is shown in Fig. 6.

MacoPharma Theraflex MB-plasma system

MacoPharma's Theraflex MB requires 1 µM MB and 180 J/cm^2 illumination dose using low-pressure sodium lights with a peak wavelength of 590 nm for inactivation. Since MB cannot permeate leukocytes, the Theraflex MB disposable is offered in two formats: if the plasma has already been membrane filtered, the set does not include a leukoreduction filter. Alternatively, if the plasma has not been leukoreduced, the disposable incorporates a leukoreduction filter. Both configurations include a filter for removal of MB and photoproducts.[84] This process is shown in Fig. 7.

Octapharma octaplas® plasma system

S/D treatment of plasma is performed by adding 1% (w/w) tri(n-butyl) phosphate (TNBP) and 1% (w/w) octoxynol-9

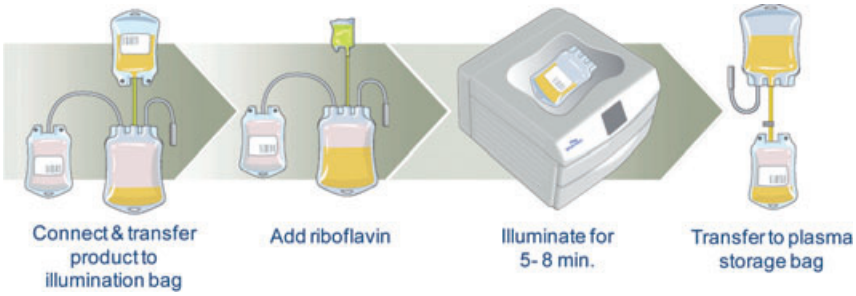

Fig. 6. CaridianBCT Biotechnologies Mirasol plasma system.

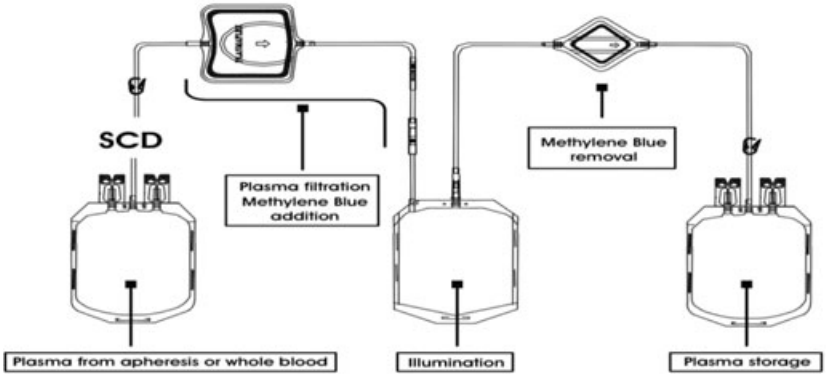

Fig. 7. MacoPharma Theraflex MB-plasma system.

TABLE 5. Log_{10} pathogen reduction for known or potential transfusion-transmitted viruses in plasma

Virus	INTERCEPT[83]	Theraflex MB[85,86]	Octaplas[81,82]
Enveloped			
HIV-1			
• Cell-free	>6.8	≥5.45	≥7.2
• Cell-associated	>6.4		
HBV	>4.5 CID_{50}*		≥6.0 CID_{50}
HCV	>4.5 CID_{50}		≥5.0 CID_{50}
HTLV-I, cell-associated	≥4.5		
HTLV-II, cell-associated	>5.7		
CMV		≥4.08	
WNV	≥6.8	≥5.78	
CHIKV	≥7.6		
Nonenveloped			
B19V	1.8		
HAV	0†		

* Chimp Infectious Dose where 50% of the animals become infected.
† No reduction was observed.

(Triton X-100) to plasma and incubating for 4 hours at 30°C. The plasma is then subjected to oil extraction and phase separation to remove the TNBP; solid phase extraction to remove the Triton X-100; sterile filtration and aseptic filling into plastic containers; and fast freezing at ≤–60°C, followed by storage at ≤–30°C.[80] It should be noted that S/D treatment of plasma is not intended as a PR method for nonenveloped viruses. S/D plasma is manufactured by pooling approximately 600-1500 source or recovered plasma donations, respectively; it is possible that transmission of an agent may occur if that agent is resistant to the S/D inactivation process or if neutralizing antibody is not present at a sufficient titer to neutralize the challenging agent.

Pathogen reduction in plasma

Published results for pathogen reduction in plasma are provided in Tables 5 through 8. Data from abstracts or unpublished data available from studies conducted by the manufacturers are included as personal communications from the companies' representatives. Cerus (INTERCEPT) has demonstrated inactivation of >5.5 logs of *O. tsutsugamushi* in an animal model. CaridianBCT Biotechnologies (Mirasol) has evaluated inactivation of a variety of transfusion-transmitted and model viruses (enveloped and nonenveloped) and parasites. The inactivation spectrum in plasma is the same as Mirasol platelets, since the platelets are suspended in 100% plasma and plasma actually constitutes the bulk of the media in which the inactivation process is being performed (Raymond Goodrich, pers. comm., 2009). Octapharma has conducted additional recent viral inactivation studies that have shown: HIV-1 (>6.3 logs); VSV (≥7.5 logs); Sindbis (>5.4 logs); pseudorabies virus (>6.3 logs); and Herpes simplex virus-2 (>6.1 logs). Since the product is cell free, risk from cell-associated viruses is also reduced. Further studies have demonstrated a substantial immune neutralization capacity in plasma pools for the following viruses: B19V (10.8 logs); HAV (≥10.0 logs); HEV (≥9.4 logs); Coxsackievirus B6 (≥8.6 logs); HSV-1 (>11.1 logs) and poliovirus type 1 (≥10.9 logs). (Tor-Einar Svae and Marc Maltas, pers. comm., 2009); Immune neutralization occurs when an antibody specific for an infectious agent binds to that agent and renders it noninfectious, but such neutralization cannot always be relied on because of the unknown prevalence of specific antibodies in the donor population.

RED BLOOD CELL PATHOGEN REDUCTION SYSTEMS

CaridianBCT Biotechnologies is using a photochemical process for treating whole blood which incorporates

TABLE 6. Log_{10} reduction of model viruses in plasma

Virus	Model for	INTERCEPT[83]	Theraflex MB[85,86]	Octaplas[81,82]
Enveloped				
Vesicular stomatitis	Enveloped viruses		>4.9	≥8
Influenza A (H5N1)	Influenza A viruses	>5.7	≥4.4	
Duck HBV	HBV	4.4-4.5	≥6	≥7.3 ID_{50}
Bovine viral diarrhea	HCV	≥6.0	≥5.44	≥6.1
Sindbis	HCV			≥6.9
Pseudorabies virus	CMV, HSV		≥5.48	≥7
Infectious bronchitis virus	Coronaviruses		4.9	
Herpes simplex virus-2	Enveloped viruses			>6.1
SARS		≥5.5		
Nonenveloped				
Bluetongue	Nonenveloped viruses	5.1		
Calicivirus	Nonenveloped viruses		≥3.9	
Human adenovirus 5	Nonenveloped viruses	≥6.8	≥5.33	

TABLE 7. Log_{10} reduction of bacteria and spirochetes in plasma

	INTERCEPT[83]
Gram Negative	
Klebsiella pneumoniae	≥7.4
Yersinia enterocolitica	>7.3
Anaplasma phagocytophilum (HGA) agent	>4.2
Gram Positive	
Staphylococcus epidermidis	>7.3
Spirochetes	
Treponema pallidum	>5.9
Borrelia burgdorferi	>10.6

TABLE 8. Log_{10} reduction of protozoa in plasma

	INTERCEPT[83]	Theraflex MB
Plasmodium falciparum	≥6.9	
Trypanosoma cruzi	>5.0	>4.9 to >5.8[87]
Babesia microti	>5.3	

riboflavin and UV light that is similar to their Mirasol process for platelets and plasma. This product is currently being tested in clinical trials in the US under an FDA-approved investigational device exemption (IDE) (Raymond Goodrich, pers. comm., 2009).

Cerus Corporation is designing a process for RBCs that uses a chemical cross-linker, specific for nucleic acid. Cerus' product entered a Phase III trial several years ago in the US, but issues with neoantigen formation brought that trial to a halt. A redesign of the process has eliminated immunogenicity in laboratory and animal studies. A Phase I trial in healthy volunteers is underway (Lily Lin, pers. comm., 2009).

CLINICAL TRIALS/CLINICAL EXPERIENCE

Platelet clinical trials

Cerus Corporation INTERCEPT platelets have undergone examination in at least 4 clinical trials in patients requiring platelet transfusion support. In the US, Cerus also conducted radiolabel recovery and survival studies in healthy volunteers, including a study in which treated platelets were also gamma irradiated.[88] Results from these studies showed a 15-20% decrease in radiolabel recovery and survival studies in INTERCEPT platelets compared to control platelets. One of the patient trials was a bleeding time study. The bleeding time correction and time to next transfusion were not statistically different between the groups, despite a lower corrected count increment (CCI) in the INTERCEPT group.[89]

INTERCEPT platelets were evaluated in thrombocytopenic patients in a two-arm, double-blind clinical Phase III-like trial in Europe using buffy coat platelets (*euro-SPRITE*).[90] One hundred three (103) subjects were enrolled in this trial, 52 patients received 311 test transfusions, and 51 patients received 256 control transfusions. Patients who received test platelets showed no statistically significant differences in their CCI compared to controls at 1 hour; however, the CCIs at 24 hours did become significantly different. There was an insignificant decrease in time between transfusions (3.0 vs. 3.4 days, test vs. control, respectively). Clinical hemostasis, hemorrhagic, and aggregate adverse events were similar between groups. Cerus also conducted a smaller pilot study in Europe that showed that 7-day INTERCEPT platelets were well tolerated and prevented bleeding. Noninferiority in terms of 1-hour CCI could not be demonstrated with a prespecified noninferiority margin of 2200.[91]

In the US, Cerus conducted a large (n = 645) Phase III double-blind, two-arm trial (INTERCEPT vs. untreated platelets) in thrombocytopenic patients using apheresis platelets (*SPRINT*). Clinical efficacy as measured by incidence of WHO bleeding Grade 2, 3, and 4 was not different between the two groups. However, 1-hour CCI, days to the next platelet transfusion, and number of platelet transfusions were statistically different, all favoring the control group.[92] This finding reflected a difference in the mean platelet dose per transfusion in the two

groups; the INTERCEPT group patients received a higher proportion of doses $<3.0 \times 10^{11}$. There was a statistically significant reduction in transfusion reactions in those patients receiving the INTERCEPT platelets compared to the control (3 vs. 4.1%). However, there was an increase in three specific pulmonary events in the INTERCEPT group: acute respiratory distress syndrome (ARDS), pneumonitis not otherwise specified, and pleuritic chest pain. Reanalysis of this apparent increase of pulmonary adverse reactions with INTERCEPT transfusions by both the study investigators and independent experts blinded to patient treatment did not confirm this difference. The original observations were attributed to inconsistent reporting of ARDS from SPRINT study sites and characteristics of the classification system used.[93,94]

CaridianBCT Biotechnologies Mirasol Platelets have been tested in three clinical trials. The company studied in vivo recovery and survival of platelets in normal volunteers and found an approximate 25% decrease in recovery and survival in the Mirasol group.[95] Results of a Phase III-like clinical trial at six blood establishments and six hospitals in France were reported at the 2008 AABB Annual Meeting.[96] In this study, efficacy data were analyzed on 80 subjects who received Mirasol-treated (test) or control apheresis platelets. Patients who received test platelets showed a statistically significant ($p < 0.002$) lower 1-hour CCI compared to control platelets. However, the CCIs were not statistically different at 24 hours. There was also a statistically shorter time between transfusions during the period of the first eight transfusions in the test group compared to the control; this difference was not observed beyond the eighth transfusion. The number of platelet transfusions per patient, total platelet dose, platelet transfusion per day of support, percent refractory patients, the number of red cells per patient, the number of WHO Grade 2, 3 and 4 bleeding events, and serious adverse events were not statistically different between the groups ($p > 0.05$). No neoantigenicity was observed.

MacoPharma has completed preclinical studies of its Theraflex UV process; it is currently under evaluation in Phase I studies.

Platelet clinical experience

To date, over 200,000 Cerus INTERCEPT platelet doses have been transfused in routine clinical use. An ongoing postmarketing observational hemovigilance program has been established to monitor the safety profile of INTERCEPT platelets. Two reports representing over 12,500 transfusions of INTERCEPT platelets to 2051 patients in 11 European centers in five countries from October 2003 to January 2007 demonstrated good clinical tolerance and a safety profile similar to untreated platelets. No episodes of transfusion-related acute lung injury or transfusion-associated graft-versus-host disease (TA-GVHD) were reported. Furthermore, the use of 65% additive solution to replace plasma in this platelet PR system may have contributed to a lower rate of acute transfusion reactions.[97,98] In a study completed in 2006 at the Blood Transfusion Center, Mont Godinne, Belgium, blood component usage was evaluated in two 3-year blocks, one before implementation of INTERCEPT and one after, in 688 and 795 patients, respectively. Primary diagnoses were similar in the two study groups: hematology (approximately 40%), oncology (approximately 6%), cardiovascular surgery (approximately 30%), and other surgery and general medical services (approximately 20%). Red cell and platelet usage was statistically unchanged before and after implementation of INTERCEPT.[99]

In a separate study, the routine use experience from two periods in EFS-Alsace, France was compared, one before implementation of platelet additive solution and INTERCEPT and one after, in 2050 and 2069 patients, respectively. In both periods, patients were transfused according to conventional medical indications. The results show that transfusion of INTERCEPT platelets to a broad patient population for a spectrum of indications was well tolerated. The incidence of adverse events was less than untreated platelet components suspended in plasma. No increase in the total platelet dose and RBCs transfused to patients was observed.[100]

Two additional clinical studies were conducted per country specific requirements: one in Luebeck, Germany[101] and the other in Basel, Switzerland.[102] Physicians ordered INTERCEPT platelets according to standard clinical practice. However, INTERCEPT platelets were used in place of gamma irradiation for prevention of TA-GVHD. In the Luebeck study, 560 INTERCEPT transfusions were administered to 52 patients with hematological malignancies. In the Basel study, 551 INTERCEPT platelet components were administered to 46 patients of whom 38 were hematology-oncology patients. The results of these studies show low rates of overall acute platelet transfusion reactions. No bleeding complications were attributable to the INTERCEPT platelets. Results of an investigator study involving 500 INTERCEPT platelet transfusions in 83 pediatric hematology-oncology patients in a routine clinical setting were reported.[103] This study showed that transfusion of pediatric patients with INTERCEPT platelets was well tolerated and provided therapeutic count increments.

As part of CaridianBCT Biotechnologies Mirasol Evaluation Program, several hundred Mirasol-treated platelet products have been transfused in routine use. No Mirasol-related adverse reactions have been reported at any of the participating blood centers or hospital sites. This program has been expanded considerably in 2009 and is targeting several thousand transfusions which will

be monitored for adverse event reporting (Raymond Goodrich, pers. comm., 2009).

Plasma clinical trials

INTERCEPT plasma has been evaluated in clinical studies in the US. In their first human clinical study, subjects donated plasma with half of the plasma treated with the INTERCEPT system and half prepared as standard fresh frozen plasma (FFP). Subjects then received warfarin over 4 days to lower the Factor VII levels. On day 4, subjects received either their own standard FFP or INTERCEPT-FFP. After 2 weeks, subjects underwent an identical protocol and received the other type of FFP. Factor VII kinetics were the same in subjects after either INTERCEPT FFP or standard FFP.[104] The efficacy and safety of INTERCEPT plasma in patients with congenital coagulation factor deficiencies was evaluated in a single-arm open-label clinical trial. The results of this 34-patient trial with deficiencies of coagulation factors I (fibrinogen), II, V, VII, X, XI, and XIII demonstrated that INTERCEPT plasma provided coagulation factor recovery and pharmacokinetics comparable to conventional plasma, with prothrombin time (PT) and activated partial thromboplastin time (aPTT) responses sufficient for adequate hemostasis.[105] Furthermore, in a randomized, double-blind clinical trial in 121 patents with acquired coagulopathy, Mintz et al. demonstrated that INTERCEPT-FFP supported hemostasis similar to conventional FFP, with no differences in the use of blood components, clinical hemostasis, or safety.[106] In a Phase III trial, Cerus evaluated the safety and effectiveness of INTERCEPT-FFP compared to standard FFP in a small group of patients with thrombotic thrombocytopenic purpura (TTP). Remission was achieved in 14 of 17 (82%) patients receiving INTERCEPT-FFP, and 16 of 18 (89%) patients receiving standard FFP. Time to remission, relapse rates, time to relapse, total volume, and number of FFP units exchanged were not significantly different between both groups. No antibodies to amotosalen were detected.[107] A hemovigilance program similar to that established for INTERCEPT platelets is ongoing to document and monitor the safety of INTERCEPT plasma transfusion.[108]

Methylene blue processes similar to MacoPharma Theraflex MB have been used in Europe for over 10 years, with over 4 million units transfused in various clinical settings. However, there have been no large controlled, randomized clinical trials comparing MB-FFP to standard FFP. Most patient studies have been small and/or used laboratory rather than clinical endpoints.

CaridianBCT Biotechnologies has evaluated their Mirasol-treated plasma in several *in vitro* studies and it has been demonstrated to meet the 14th Edition, Council of Europe Guidelines for protein content for standard FFP (Raymond Goodrich, pers. comm., 2009).

Octapharma octaplas has been evaluated in several uncontrolled, observational trials in liver transplant, cardiac surgery, and TTP patients, with no differences noted between S/D plasma and FFP. There have been a few randomized, controlled clinical trials in patients with severe coagulopathy or undergoing cardiopulmonary bypass surgery, with no clinical differences noted. Whether these studies were sufficiently powered to see any differences is debatable.

Plasma clinical experience

Over 6 million units of Octapharma octaplas have been transfused, and it has been accepted as therapeutically equivalent to standard FFP. No postmarketing hemovigilance trials have been published.

MacoPharma Theraflex MB has been registered or is in routine use in 20 countries, including Germany, Switzerland, Spain, Greece, Italy, France, Belgium, and the UK. In spite of laboratory observations demonstrating some loss of coagulation factors, clinical reports have been generally satisfactory. Postmarketing hemovigilance studies from Greece and Spain evaluating 8500 units and 88,000 units, respectively, have been reported, with no adverse events associated with MB-FFP and satisfactory clinical outcomes.[109,110] Castrillo et al.[111] also reported a 5-year experience with MB plasma with no adverse reactions observed. MB-FFP in TTP patients has been evaluated in several small studies, comparing MB-FFP to untreated FFP and its effectiveness is a subject of debate. Two studies in Spain, one retrospective and one prospective, demonstrated that there was a lower remission rate and higher volume of FFP required for treating TTP when using MB-FFP compared to control (untreated) FFP.[112,113] It has been hypothesized that this is due to decreased levels of ADAMTS13, the enzyme generally thought to be deficient or inhibited in these patients; however, MB treatment does not affect the activity of ADAMS13.[114,115]

Limitations of pathogen reduction

Agents with intrinsic resistance to PR processes include prions, some nonenveloped viruses such as HAV, and bacterial spores. Furthermore, extraordinarily high-titer viruses like B19V or HBV may not be inactivated below an infectious dose. Therefore, surveillance for the emergence of new agents will remain critical even after the introduction of PR.

Since blood components currently carry very low risks of infectious agent transmission, any manipulation to further mitigate known risks will be difficult to justify if it introduces any material new risk to transfusion recipients. Critical questions have been raised about

short- and long-term safety of PR systems to transfusion recipients, to blood center and hospital staff who may be exposed to them, and to the environment in which they are manufactured, used, and in which they are disposed.

Potential toxicologic effects of the candidate PR systems have been examined in many dimensions. These include acute, subacute, and chronic toxicity, blood component incompatibility, genotoxicity, carcinogenicity, and impact on reproduction and development.

In general, the residual levels of active PR ingredients in fully processed blood components are below the limit of detection in available direct toxicity assays due to robust removal steps included in the processes. Also, water-soluble molecules are rapidly excreted with no accumulation in fat. There are, however, diverse reaction products derived from the active agents and establishing large safety margins for these products is difficult due to dose per volume constraints (John Chapman, pers. comm., 2009).

Compatibility with cellular elements and plasma proteins is critical. Neoantigenicity with sensitization to treated components is one aspect. Another is the impact on the recovery, survival, and function of blood elements. It is important to understand whether or not PR will increase transfusion requirements related to loss of therapeutic product, or if processing disturbs the delicate balance in physiologic pathways like coagulation.

All PR methods in development for cellular blood products rely on interactions with microbial nucleic acids, raising the specter of carcinogenicity and mutagenicity, and of adverse impacts on reproduction and development. The study of these potential effects is very difficult, expensive, and time-consuming. While in vitro and animal studies can be completed with reasonable speed and economy and are reassuring when negative, the clinical events of interest are expected to be quite rare and may have very long latent periods until recognition. This raises barriers on the path toward regulatory approvals. Special, more vulnerable populations may need additional focus in clinical studies. These include pregnant and fertile women, the fetus, newborns, and growing children, those requiring massive acute transfusions, and patients who receive chronic transfusion support. There needs to be a level of comfort that those with impaired kidney and liver function are not at elevated risk of adverse events.

Although the companies have been authorized to market their products in many countries (Table 9), adoption and routine use of these technologies has been relatively limited. Blood centers, hospitals, and transfusion services will continually evaluate the cost versus benefit of each of these technologies; at the present time, the most efficient and effective process(es) is unknown.

PATHOGEN REDUCTION AND ITS POTENTIAL FOR EID AGENTS

The potential utility of PR can be appreciated by reviewing the list of 16 agents assigned red, orange, or yellow priority in this exercise. The data cited, either for specific agents or relevant models, suggest that only the vCJD prion of the red agents, and the CWD prion, human parvovirus B19, and HAV among the others, will likely escape inactivation from clinically relevant titers in platelets and plasma with application of the systems being brought forward. The data available for RBCs are not adequate to instill great confidence at this point, but early data certainly support concentrated research efforts. While some see the absence of a single process applicable to all components as a barrier to the use of PR, just the prospect of controlling bacterial contamination of platelets, the most common serious infection associated with contemporary blood transfusion in the developed world, is an example of its potential power. Pathogen reduction would be a useful intervention to reduce transfusion transmission of the agents responsible for babesiosis, Chagas disease, and malaria. Babesiosis from RBC transfusion is widely understood to be more prevalent than published reports have suggested, and antibody to *T. cruzi* testing has been adopted nearly universally in the US while emerging evidence demonstrates that *T. cruzi* transmission may be less common than had been anticipated when decisions were made to pursue donor testing. Deferral for minimal risk of transfusion-transmitted malaria remains a source of serious donor loss. A rational approach to testing for these three parasites may be selective screening strategies, but less than universal screening is a strong disincentive for test builders to bring a donor screening assay through the rigorous and costly regulatory approval process; thus, market forces may prevent or delay test development. Potentially, PR bypasses this "one agent-one test" approach while protecting both recipients and the donor supply. Similarly, if the next agent to emerge as a serious threat is an enveloped virus like HBV, HIV, HCV, and WNV, it is probable that the approaches in development will be robust, prospectively obviating the need for deferrals and testing. It is also anticipated that a thorough review of the currently used donor screening questions and tests will be required to assess their continued need once robust pathogen reduction methods are available. Similarly, well-controlled postmarketing studies will be needed to determine if adverse outcomes occur as a result of widespread use of PR and the impact of PR on transfusion-transmitted disease.

CONFLICT OF INTEREST

Peyton S. Metzel is an employee of Fenwal, Inc. Fenwal, Inc. manufactures InterSol platelet additive solution and

TABLE 9. Development/regulatory status

	Platelets			FFP			
Regulating agency or development status	INTERCEPT	Mirasol	Theraflex UV	INTERCEPT	Mirasol	Theraflex MB	Octaplas
Development Status, US	Phase III Completed	Phase I Completed		Phase III Completed			Phase III Completed
Development Status, Europe	Completed	Completed	Phase I	Completed	Completed	Completed	Completed
US FDA							
Canadian MOH						✓	
CE Mark or MAA*	CE	CE	CE	CE	CE	CE	MAA
For sale In:							
• Argentina						✓	✓
• Australia						✓	
• Austria							✓
• Belgium	✓	✓		✓	✓	✓	✓
• Brazil						✓	✓
• Canada							✓
• Croatia							✓
• Czech Republic	✓			✓			✓
• Finland	✓	✓		✓	✓		✓
• France	✓			✓		✓	✓†
• Germany	✓					✓	✓
• Greece	✓			✓		✓	
• Gulf Central Committee							✓
• Hungary	✓			✓			✓
• Iceland	✓	✓			✓		✓
• Ireland	✓						✓
• Italy	✓	✓		✓	✓	✓	✓
• Kazakhstan		✓			✓	✓	
• Kuwait	✓	✓			✓		✓
• Luxembourg		✓			✓		✓
• Malaysia	✓			✓		✓	
• Mexico							✓
• Netherlands							✓
• New Zealand							✓
• Norway	✓	✓		✓	✓		✓
• Oman							✓
• Poland	✓	✓		✓	✓	✓	
• Portugal	✓	✓		✓	✓		✓
• Romania							✓
• Russia	✓			✓		✓	
• Saudi Arabia		✓			✓		✓
• Singapore	✓					✓	
• Slovakia	✓	✓		✓	✓		✓
• Slovenia	✓			✓			✓
• Spain	✓	✓		✓	✓	✓	
• Sweden	✓	✓		✓	✓		✓
• Switzerland							✓
• Thailand	✓			✓	✓		
• Turkey	✓						
• UAE		✓					✓
• UK						✓	✓
• Vietnam	✓						
• Yemen							✓

* Council of Europe approval or Marketing Authorization Approval. A CE Mark alone is not adequate regulatory authority to market a device in many countries in Europe including the UK, France, and Germany.

† SD-plasma available, similar to Octaplas.

is the contract disposables manufacturer for Cerus Corporation INTERCEPT platelets and plasma. No other conflicts of interest were declared.

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Efficacy of apheresis platelets treated with riboflavin and ultraviolet light for pathogen reduction

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BACKGROUND: Pathogen reduction technologies for platelet (PLT) components offer a means to address continued viral transmission risks and imperfect bacterial detection systems. The efficacy of apheresis PLTs treated with riboflavin (vitamin B2) plus ultraviolet (UV) light (Mirasol, Navigant Biotechnologies) was investigated in a single-blind, crossover study in comparison to untreated PLTs.

STUDY DESIGN AND METHODS: Normal subjects (n = 24) donated PLTs by apheresis on two occasions at least 2 weeks apart. Units were randomized to control or test arms, the latter receiving the addition of 28 mL of 500 μmol per L B2 and exposure to 6.2 J per mL UV light. PLTs were stored for 5 days with biochemical and hematologic analyses performed before and after illumination on Day 0 and at the end of storage. An aliquot of each unit was radiolabeled and returned to determine recovery and survival.

RESULTS: The PLT content of treated units was maintained from Day 0 ($4.1 \times 10^{11} \pm 0.4 \times 10^{11}$) to Day 5 ($4.0 \times 10^{11} \pm 0.4 \times 10^{11}$). Treatment with B2 plus UV light was associated with an increase in lactate production with concomitant increases in glucose consumption. pH (control, 7.38 ± 0.07; test, 7.02 ± 0.10) was well maintained throughout storage. Recovery of treated PLTs (50.0 ± 18.9%) was reduced from that of control PLTs (66.5 ± 13.4%); survival was similarly shortened (104 ± 26 hr vs. 142 ± 26 h; $p < 0.001$).

CONCLUSIONS: PLTs treated with B2 plus UV light demonstrate some alterations in in vitro measures but retain in vitro and in vivo capabilities similar to pathogen-reduced and licensed PLT components that have been shown to have useful clinical applicability. The recovery, survival, and metabolic properties of Mirasol PLTs should provide sufficient hemostatic support in thrombocytopenia to justify patient clinical trials.

Advances in donor screening and testing have reduced the risks of transmission of human immunodeficiency virus and hepatitis C virus by more than 10,000-fold in the past two decades.[1] Diversion and detection methods have been introduced to reduce the risks of bacterial contamination in platelets (PLTs).[2] Despite these efforts, risks of viral and bacterial contamination remain. Furthermore, various parasitic risks have yet to be addressed successfully, including those of Chagas disease, and the evolution or introduction of new viruses into the blood supply may cause significant morbidity and mortality before their recognition can lead to the development and implementation of effective testing.[3] Therefore, important health benefits may accrue with the introduction of pathogen reduction technologies (PRTs) in blood components.

Many recipients of PLT transfusions are especially vulnerable to the presence of contaminating pathogens. Their immunosuppressed and/or neutropenic states may blunt effective response to a pathogen. Although some recipients of PLT transfusions may succumb to their

ABBREVIATIONS: B2 = riboflavin (vitamin B2); PRT(s) = pathogen reduction technology (-ies).

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underlying disorder, the success of more effective treatments for malignancies over the past several decades is eliminated if the patient dies because of a transfusion-transmitted infection. Thus, the means to reduce the infectivity and pathogenic potential of contaminating organisms in PLT components remains an important goal.

The success of a proposed PRT may be judged in terms of safety and efficacy.[4] The treatment must not, in itself, create more risk than it is removing, and it must reduce the presence of viable or infective pathogens to a clinically useful degree. Similarly, the transfusible component must be well tolerated and retain a sufficient proportion of its efficacy (viability and thrombogenicity in the case of PLTs) to achieve the clinical aims of the transfusion.

Riboflavin (vitamin B2) has been shown to interact with genomic material and irreversibly, covalently combine with RNA and DNA to preclude transcription and translation of pathogens' genetic material on exposure to ultraviolet (UV) light and/or to cause breakage of the chromosomal strand.[5-8] The process has been shown to be effective against viruses, bacteria, and parasites with sufficient reductions in infectivity (generally ≥5 log) to provide a useful safeguard for transfusion recipients.[9-14] The low toxicologic concern regarding use of a vitamin to achieve this task has been bolstered by embryofetal, subchronic, and high-dose toxicology studies; pharmacokinetic analysis; neoantigenicity, cytotoxicity, and hemocompatibility experiments; chromosome aberration and Ames tests; and a mouse micronucleus mutation assay, all of which failed to demonstrate results suggestive of human toxicity.[15-20] In vitro analyses of treated, plasma-suspended PLTs indicated good retention of biochemical and functional variables through 5 days of storage following treatment.[21,22]

To move investigation of the effect of treating PLTs with B2 and UV light to the next stage, we conducted a crossover study in blinded normal subjects to determine the in vivo efficacy of treated versus control (untreated) PLTs after 5 days of storage via autologous recovery and survival studies of radiolabeled PLTs.

MATERIALS AND METHODS

The study received the approval of local institutional review boards at each participating location and was conducted under an investigational device exemption from the FDA. Twenty-nine normal, healthy adult subjects (20 men and 9 women, all without reproductive potential) who met all FDA and AABB[23] criteria for PLT donation pertaining to the health of the donor were recruited into the study at two locations (15 at one site; 14 at the other) without regard to previously obtained recovery and survival results. Written informed consent was obtained from subjects. Five subjects did not complete the study owing to inadvertent duplicate illumination of the PLT component, intercurrent illness (influenza), venipuncture difficulties, psychiatric hospitalization (owing to an undisclosed existing condition), and incomplete sample collection; a total of 24 paired data sets (17 men, 7 women) were thus generated.

Subjects donated 1 unit of leukoreduced apheresis PLTs on an automated blood collection system (Trima Accel, Gambro BCT, Lakewood, CO) on two occasions at least 2 weeks apart with a target of 1640×10^3 PLTs per µL in 270 mL of plasma–acid citrate dextrose (ACD)-A. PLTs were placed in ELP bags composed of polyvinyl chloride plasticized with *N*-butyryl tri-*n*-hexyl citrate.[24] Units were held undisturbed immediately after collection for 2 to 3 hours to allow dissociation of any PLT aggregates. The PLT content of each unit was verified to be in the target range of 1400×10^3 to 1880×10^3 PLTs per µL 2 to 4 hours after collection; if necessary, the concentration was reduced to within the allowed range with some of the 150 mL of plasma that had been collected at the time of the plateletpheresis. The protocol allowed for a repeat collection if any unit failed to meet the content criterion or other problems developed that precluded successful analysis and return of the unit.

Units were designated to undergo treatment with B2 plus UV light (Mirasol, Navigant Biotechnologies, Lakewood, CO; treated arm) or not (control arm) according to a statistical package randomization scheme provided by the sponsor. (If subjects were withdrawn from the study, they were replaced by subjects who assumed their place in the randomization table.) At 2 to 4 hours after collection, a volume of 250 ± 5 mL (256 g) of PLTs was gravimetrically transferred from the ELP collection bags to the PLT illumination-storage bag to be either Mirasol-treated or stored without further processing (control). B2 solution (500 µmol/L, 28 ± 1 mL) was added to the test unit through a sterile barrier filter with a syringe. After thorough mixing, a 3- to 5-mL sample was withdrawn for in vitro assessments. The unit was then secured in a prototype illumination device and exposed to 5.0 J per cm^2 of UV light (corresponding to 6.2 J/mL delivered to the PLT unit), a process that took 8 to 10 minutes in the temperature-controlled environment of the illuminator (illuminating wavelengths, 265-370 nm). After the process, a 3- to 5-mL postillumination sample was obtained from the test unit for in vitro assessments. One 3- to 5-mL sample was obtained from the control product for in vitro assessments. The units (control and treated) were stored in the same PLT illumination-storage bag under normal blood banking conditions of 22 ± 2°C with horizontal agitation for 5 days. On Day 3 or Day 4, a 3- to 5-mL sample was taken for bacterial culture to document sterility.[25] At the end of the 5-day storage period, a 3- to 5-mL sample was withdrawn from the products for in vitro assessments and radiolabeling. (All sampling was conducted with a sterile

connecting device. All unit handling and processing was performed at each test site.)

PLTs were labeled with standard techniques before return into the original donor with standard techniques.[26,27] A 10-mL aliquot was removed from the unit, and the PLTs were concentrated by centrifugation. Approximately 100 µCi of the radiolabel (^{111}In-oxine, Medi-Physics, Arlington Heights, IL) were added to a suspension of the PLTs for a 20-minute room temperature incubation. The PLTs were then washed with a mixture of plasma and ACD-saline. By use of a dose calibrator, 10 to 20 µCi of the labeled PLTs was taken up into a syringe for return into the subject. (The remainder was used for standards and quality control procedures.) Samples were taken from the contralateral arm within the first 3 hours after return as well as daily for 1 week (for determination of the proportion of injected label recoverable) and again on Day 10 (to allow for correction of activity associated with red blood cells). ^{111}In emissions were counted at 176 to 190 keV. Recovery was calculated from the injected radioactivity (corrected for preinjection radiolabel elution[27]), a formulaic projection of the subject's blood volume,[27] and back-extrapolation to the time of injection with the multiple-hit model on a standardized computer program[28,29] and verified by a separate calculation with a validated computer program (SAS Institute, Inc., Cary, NC). Survival was recorded as the mean residual life span determined by the multiple-hit model.

In vitro analyses were conducted on Day 0 (before treatment in the test units) and on the last day of storage. PLT concentration was determined from a CBC assessment with a hematology analyzer (Advia 120, Bayer Diagnostics, Tarrytown, NY; or 9110-plus Baker Instruments, Allentown, PA). For this purpose, a sample was transferred to an ethylenediaminetetraacetate tube, which was rotated at 22 ± 2°C until the sample was analyzed. The lactate and glucose concentrations were determined on an automated lactate-glucose analyzer (either the ABL-705, Radiometer-America, Westlake, OH; or the Hitachi 917, Hitachi High-Technologies, Tokyo, Japan). The rates of lactate production and glucose consumption during the 5 days of storage were calculated with a concentration normalized to the initial PLT concentration. Unit pH was analyzed on an automated blood gas analyzer (ABL-705, Radiometer-America; and Model 855, Bayer Diagnostics) at 37°C and converted to $pH_{22°C}$ algebraically. O_2 and CO_2 partial pressures were analyzed on an automated blood gas analyzer (ABL-705, Radiometer-America; or Model 248 or 855, Bayer Diagnostics). Expression of CD62 or GMP-140 (P-selectin) was determined by flow cytometry within 14 days after fixation in 1 percent paraformaldehyde per the method recommended by the Biomedical Excellence for Safer Transfusion (BEST)[30] based on fluorescence intensity. PLT morphology was characterized with a visual swirl scoring technique with a three-tiered scoring system: positive swirl (inhomogeneity readily visible throughout the entire bag, 3), intermediate swirl (some inhomogeneity visible, 1), and negative swirl (turbid, 0).

Results are expressed as means ± 1 standard deviation (SD). Statistical analysis was conducted comparing control versus treated units with a t test statistic with a p value of less than 0.05 used to reject the null hypothesis of no difference. Paired analyses were applied wherever appropriate.

RESULTS

All subjects participating in the study had no adverse events related to their participation. All PLT returns were well tolerated. All unit cultures were negative. Ten repeat apheresis procedures were performed in nine subjects. Five repeat apheresis collections were required in the experimental arm and four were required in the control arm owing to factors such as intercurrent illness precluding return and handling of units in manners not consistent with the study design. One apheresis procedure had to be aborted because of infiltration.

The PLT concentrations were 10 percent lower in the treated units reflecting the dilution caused by the addition of the B2 but total PLT counts remained unchanged (Fig. 1). In addition, there was no difference in PLT count between before treatment to the end of storage in the treated units.

Treated units demonstrated accelerated glycolysis with lower glucose concentration at the end of storage, higher lactate concentration, and increased rates of consumption of glucose and production of lactate (Figs. 2A and 2B). There was, however, residual glucose in all units at the end of the storage period, and the pH value, although lower in treated units, remained above 6.8 in all units (Fig. 2E). Blood gas measurements showed a decrease in pO_2 and pCO_2 during the storage period

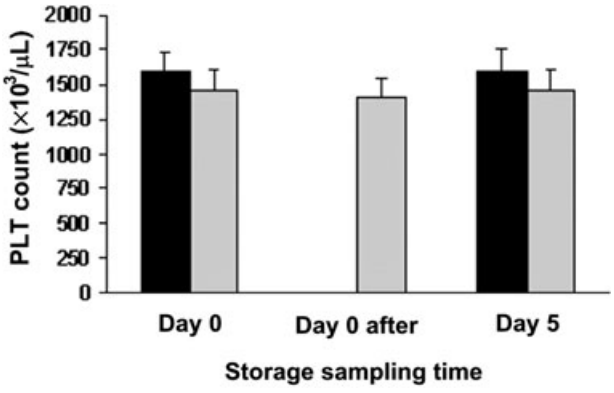

Fig. 1. PLT concentration. Results of testing before and after treatment (Day 0) and at end of storage (Day 5). (■) Mean of control units; (□) treated units. Vertical bars represent 1 SD.

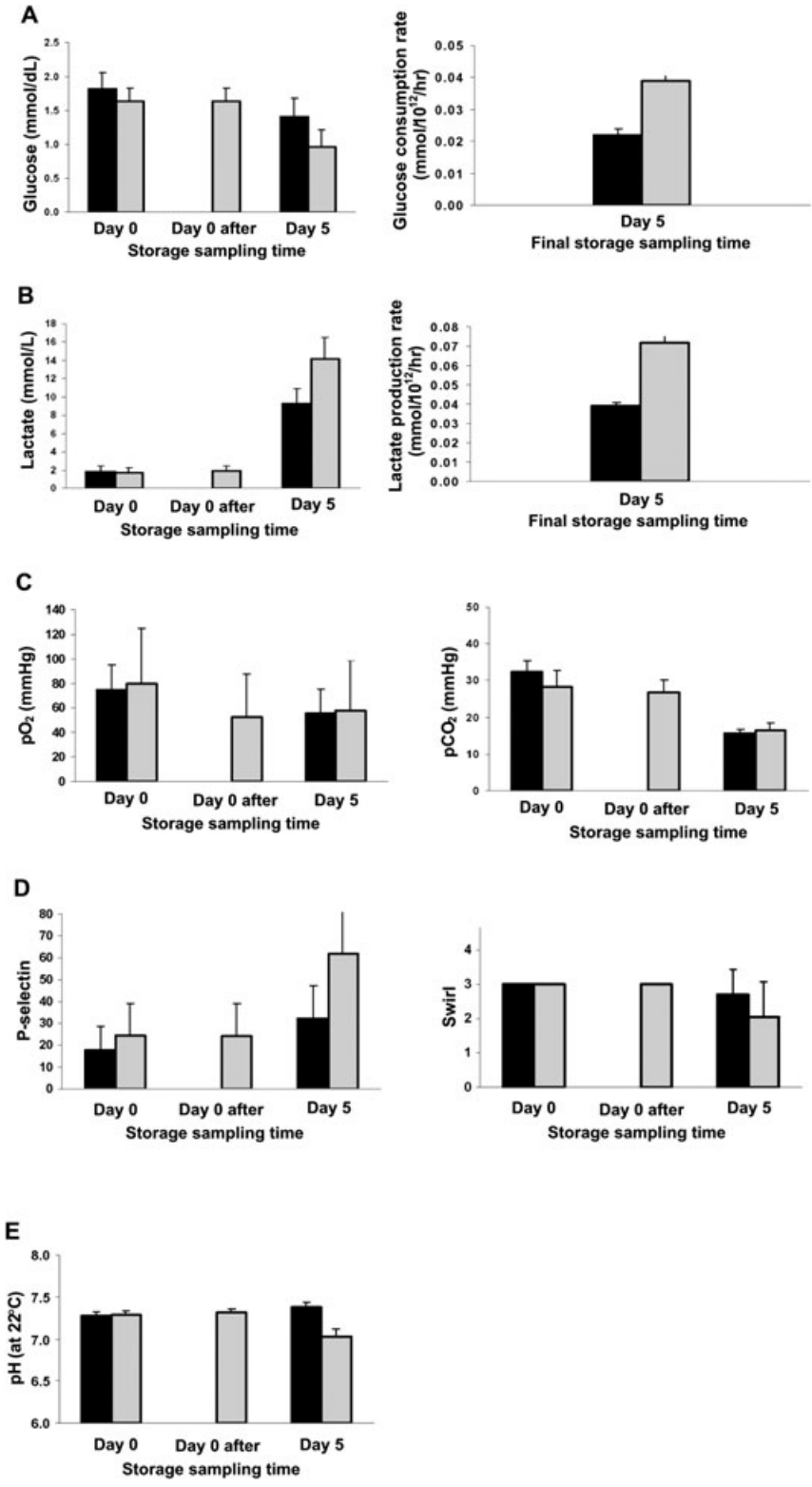

Fig. 2. Results of testing before and after treatment (Day 0) and at end of storage (Day 5). (■) Mean of control units; (□) treated units. Vertical bars represent 1 SD. (A) Glucose concentration and consumption rate. (B) Lactate concentration and consumption rate. (C) Oxygen and carbon dioxide partial pressures. (D) P-selectin expression and swirling. (E) Unit acidity.

(Fig. 2C). The Day 0 and Day 5 results were not different between control and treated units' pO_2; pCO_2 was significantly lower in test units before treatment but higher at the end of storage. P-selectin expression increased throughout storage in both groups of units and was higher on Day 5 in test than control units (Fig. 2D). The assessment of swirling was not different at the beginning of storage but was lower on Day 5 in treated units. The differences in in vitro measures between control and treated units at the end of the storage period (as shown in the figures) were all significant except for pO_2 and pCO_2.

Radiolabel was taken well by both test and control PLTs on Day 5 (Table 1). The recovery of radiolabeled PLTs from two subjects' control units exceeded biologic possibility (i.e., recovery >100%) for reasons that could not be determined. These control units' in vivo results were excluded from further analysis, but the treated units' results were included in (unpaired) analyses. The mean recovery and survival of treated PLTs were reduced when compared with the recovery and survival of the control units (Table 1).

Possible relationships between in vitro measures and in vivo PLT recovery were analyzed by means of linear regression across both types of units (Table 2). The best correlations with in vivo recovery were seen for the rate of glucose consumption ($p = 0.0000$), lactate concentration ($p = 0.0000$), rate of lactate production ($p = 0.0001$), pH ($p = 0.0002$), glucose concentration ($p = 0.0031$), and P-selectin ($p = 0.0078$). The in vitro variable that correlated most closely with survival was Day 5 pH ($p = 0.0021$). With stepwise multiple regression, recovery and survival were each fitted against all in vitro variables. It should be noted that there are strong correlations between many of the in vitro variables themselves, which has a confounding effect on obtaining a definitive model. In the case of recovery, the final regression model obtained by the stepwise regression method contained only the rate of glucose consumption. In the case of survival, pH was the only variable main-

tained in the model. When these analyses were repeated by also including treatment (control vs. treated) in the model, only the rate of glucose consumption remained as a significant predictor of recovery. In the model for survival, only treatment was significant.

DISCUSSION

This randomized crossover study in normal subjects documented that treatment of leukoreduced apheresis PLTs with B2 plus UV light to effect pathogen reduction causes a reduction in radiolabeled autologous recovery and survival but retention of sufficient in vivo efficacy to predict clinical utility. The in vitro analyses indicated that the treated PLTs were undergoing glycolysis at an accelerated rate compared to untreated controls. This metabolic substrate, however, was not exhausted during a 5-day storage period, and the acidity of the treated units, although greater than for control units, did not exceed the capabilities of the PLTs to maintain functional metabolism. One may speculate that a "hypermetabolic state" may be induced by the treatment, but the magnitude of this does not preclude continued viability of the cells. The effect of the treatment process was also seen through increased P-selectin expression and reduced swirling at the end of the storage period in comparison with untreated control units. Importantly, the PLT concentrations, although lowered in the test units by the addition of 28 mL of solution containing the B2, were well maintained through treatment and storage. Thus, although all systems that utilize UV light treatment as part of PRT appear to have negative effects on PLT physiology,[31] the Mirasol system appears to have less of an effect on the number of PLTs available remaining in the unit through the treatment process.

The reductions in recovery and survival can be assessed in several different ways. Because the laboratories participating in this study use the same radiolabeling procedure and have participated jointly in other studies, the results of this study can be usefully compared. The recoveries and survivals reported here for the control units were very similar to those reported in a study on (untreated) leukoreduced PLTs after 5 days of storage (recovery, 63.0 ± 11.2%; survival, 161 ± 38 hr)[32] giving confidence that the variability introduced into such analyses over time and over different subjects is minimal when a standardized protocol is used. Furthermore, the recovery of untreated PLTs stored for 7 days (53.9 ± 13.8%), which was adequate for FDA acceptance of approval for 7-day storage on Trima PLTs, was very close to that observed with treated PLTs stored for 5 days in this trial (50.0 ± 18.9%). (The survival of treated PLTs at 5 days of storage, 104 ± 26 hr, was shorter than that of untreated PLTs at 7 days, 134 ± 45 hr, $p < 0.05$.)

Both of these proposed modifications to PLT systems (extended storage to reduce outdating and pathogen reduction treatment to reduce infectious risks) offer useful advantages but obviously carry with them certain downsides, including reduced efficacy. A trade-off is inevitable in such situations, and value judgments will need to be made to determine whether a reduction of in vivo efficacy is mitigated by reducing the risk of certain pathogens. It is, however, encouraging to note that Mirasol PLTs at 5 days of storage appear similar to untreated PLTs at 7 days of storage, an approach that has been shown to have clinical utility.[25] Because comparison of a new approach to PLT handling (such as PRT) to a previously licensed method may lead to "creeping inferiority" via repetitive comparisons,

TABLE 1. Results of radiolabeling studies*

	Control	Treated	p value
Radiolabel uptake efficiency (%)	64.2 ± 17.2	59.5 ± 21.2	>0.05
Recovery (%)	66.5 ± 13.4	50.0 ± 18.9	<0.05
Survival (multiple hit, hr)	142 ± 26	104 ± 26	<0.05

* Results of Day 5 radiolabeling with ^{111}In and autologous return. Results shown are 22 observations from the control cycle and 24 from the treated cycle.

TABLE 2. Correlation via linear fit of Day 5 in vitro measures with recovery and survival

	Correlation with recovery			Correlation with survival		
Measure	Correlation coefficient (r)	F value	p value	Correlation coefficient (r)	F value	p value
PLT concentration	0.3235	5.1422	0.0283	0.1651	1.2327	0.2729
Glucose concentration	0.4269	9.8054	0.0031	0.3514	6.1989	0.0166
Glucose consumption rate	–0.5981	24.5106	0.0000	–0.3505	6.1619	0.0169
Lactate concentration	–0.5836	22.7236	0.0000	–0.3564	6.4036	0.0150
Lactate production rate	–0.5608	20.1895	0.0001	–0.3652	6.7707	0.0126
$pH_{22°C}$	0.5263	16.8536	0.0002	0.4426	10.7206	0.0021
pO_2	–0.2061	1.9527	0.1693	–0.0725	0.2325	0.6320
pCO_2	–0.0829	0.3047	0.5838	–0.0272	0.0325	0.8578
P-selectin expression	–0.3875	7.7734	0.0078	–0.1767	1.4179	0.2401
Swirling	0.1275	0.7270	0.3985	0.2704	3.4710	0.0691

an alternative approach to evaluating in vivo efficacy has been suggested wherein the recovery and survival of PLTs handled in the new system are compared to fresh PLTs from the same subject.[33] This proposal, which requires the "test" PLTs to demonstrate at least two-thirds the recovery and one-half the survival of fresh PLTs in the same subject, has been successfully validated as providing a realistic and reproducible benchmark.[34,35] The current study was designed before the proposal for comparison with fresh PLTs. Use of a standardized protocol for preparation of the fresh PLTs yields recoveries in the range of 60 to 65 percent and survivals approximating 200 hours.[26,36] Applying the target proportions, one would expect that acceptable performance would fall into the range of 40 to 44 percent recovery and approximately 100 hours of survival. The Mirasol-treated PLTs prepared in this study would thus appear to meet the new standard, but a direct comparison with noninferiority statistical comparison would, of course, be needed to verify this.[37]

A similar study has recently been published with amotosalen and UV light to effect PRT in PLTs.[38] A direct comparison of in vivo results would suggest that Mirasol-treated PLTs offered improved recovery and survival, although technical differences cannot be excluded as the source of this.

This study has documented the effects of applying a PRT system for PLTs that has a low potential for toxicity. The system yields PLTs that clearly show the effect of the treatment, in terms of altered biochemical analyses and reduced recovery and survival, but that retain sufficient viability and functionality to predict their clinical utility. This study therefore would suggest that clinical trials to investigate the effectiveness of Mirasol PLTs in thrombocytopenic patients are warranted.

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Assessment of the genotoxic potential of riboflavin and lumiflavin

A. Effect of metabolic enzymes

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Summary

The mutagenic potential of riboflavin and its photodegradation product lumiflavin was evaluated using the *umu* test, SOS chromotest and Ames Salmonella assay. Both riboflavin and lumiflavin by themselves were found to be non-mutagenic. On treatment with rat liver microsomal enzymes (S9) or caecal cell-free extract (CCE), lumiflavin acquired mutagenicity, while the status of riboflavin remained unaffected. Activation of lumiflavin by metabolic enzymes was found to result in an alteration of its spectral characteristics.

Riboflavin, a vitamin, has found widespread application in food products both as a nutrient and as a colouring agent (Counsell et al., 1981). Unlike many other food colourants it has commanded unquestioned favour from regulatory agencies all over the world (Horwitt, 1972; FAO, 1981), since earlier toxicological studies have failed to reveal any deleterious effects (Purchase et al., 1978; Haveland-Smith, 1981). However, evidence obtained by many other investigators has indicated that riboflavin when exposed to light could produce mutagenic (Griffin et al., 1981; Bradley and Sharkey, 1977; Pathak and Carbonare, 1988) as well as cytotoxic effects (Lee, 1969; Misra et al., 1987, 1990) in eukaryotic cells. Synergistic effects of riboflavin with light have also been shown to bring about alterations in DNA and in individual nucleotides in vitro (Uehara, 1966; Speck et al., 1976; Korycka-Dahl and Richardson, 1980; Alvi et al., 1984). These studies clearly indicated the toxic potential of riboflavin. However, the molecular mechanisms associated with the interaction of riboflavin or its photodegradation product lumiflavin with living cells are poorly understood.

In the present report an attempt has been made to assess the mutagenic potential of riboflavin as well as its photodegradation product lumiflavin using three short-term tests, namely the *umu* test, SOS chromotest and Ames/Salmonella assay.

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Materials and methods

Riboflavin (7,8-dimethyl-10-(D-ribityl) isoalloxazine) was a kind gift from Glindia Ltd. (Bombay, India). Lumiflavin (7,8,10-trimethyl isoalloxazine), 4-nitroquinoline-*N*-oxide (4NQO), 9-aminoacridine (9AA), mitomycin C (Mit C), nalidixic acid, benzo[*a*]pyrene (Bap), rutin, nicotinamide adenine dinucleotide phosphate (NADP), glucose-6-phosphate (G-6-P), L-histidine · HCl, biotin, *p*-nitrophenyl phosphate (PNPP), sodium dodecyl sulfate (SDS) and *O*-nitrophenyl-α-D-galactopyranoside (ONPG) were purchased from Sigma Chemical Company (St. Louis, MO, USA), 4-dithio-DL-threitol (DTT) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) were acquired from Fluka AG (Germany). Aroclor 1254 was obtained from Monsanto (St. Louis, MO, USA). Nutrient broth, tryptone and yeast extract were acquired from Difco Laboratories (Detroit, MI, USA). All other chemicals and solvents were of AR grade, the latter were distilled before use. The purity of riboflavin and lumiflavin was ascertained by TLC using silica gel with two different solvent systems, viz. pyridine–glacial acetic acid–water (10 : 1 : 40, v/v) and *n*-butanol–acetic acid–water (40 : 10 : 50, v/v).

Salmonella typhimurium TA100, TA98 and TA97a were kindly provided by Prof. Bruce N. Ames (University of California, Berkeley, CA, USA). *Salmonella typhimurium* TA1535/psk 1002 was obtained from Dr. Yoshimitsu Oda (Osaka Prefectural Institute of Public Health, Nakamachi-1, Japan) while *Escherichia coli* PQ37 was acquired from Dr. Maurice Hofnung (Institut Pasteur, Paris, France). Characteristics of the strains were confirmed and frozen permanents prepared according to the published protocols (Maron and Ames, 1983; Quillardet and Hofnung, 1985). Working stock cultures of Ames strains were maintained on nutrient agar slants and those of other strains on Luria agar slants and were stored at 0–4°C until use.

Preparation of liver S9 fraction

Male Wistar rats weighing 200–250 g were injected intraperitoneally (500 mg/kg body weight) with Aroclor 1254 (200 mg/ml DMSO). On the fourth day, feed was removed and on the following day the animals were killed by cervical dislocation. Livers were removed under sterile conditions into ice-cold 0.15 M KCl and S9 fraction was prepared according to the method of Maron and Ames (1983). The tissue was homogenised in 0.15 M KCl using a Potter Elvehjem glass homogeniser with 5 up and down strokes of a teflon pestle (25% homogenate). The homogenate was then centrifuged at 9000 × *g* for 10 min and the supernatant (S9 fraction) was decanted and was dispensed in 2-ml aliquots in sterile plastic tubes which were frozen quickly in liquid nitrogen and stored at −80°C until use. The S9 mixture was prepared freshly before use (Ames et al., 1975) by incorporating 4 mM NADP, 8 mM $MgCl_2$ and 33 mM KCl to a 1 : 10 diluted fraction. Dilution for SOS chromotest was carried out with Luria broth instead of a buffer. The mixture was sterilised using a 0.45-μm Millipore filter. The metabolic activity of S9 was ascertained using the Ames/Salmonella assay (Ames et al., 1975). After activation benzo[*a*]pyrene exhibited 900 additional revertants.

Caecal cell-free extract

Caecal cell-free extract (CCE) was prepared following the procedure of Brown and Dietrich (1979). Male Wistar rats weighing about 200–250 g were killed by cervical dislocation and the caecum was removed. The caecal contents were suspended in cold Krebs-Ringer phosphate buffer, pH 7, containing 2.5 mg/ml DTT for the Ames/Salmonella assay or *umu* test or in Luria broth for the SOS chromotest. The suspensions were homogenised in a Potter Elvehjem homogeniser and sonicated for 5 min (Sonics and Materials Inc.). The sonicated extract was centrifuged at 13,000 × *g* for 15 min and the supernatant was filtered through a Millipore filter (0.45 μm) and stored at −80°C until use. CCE-activated rutin showed 200 revertants/100 mg/plate with *Salmonella typhimurium* TA98.

Mutagenicity assays

(1) Umu test. The general screening of mutagenicity of riboflavin and lumiflavin was carried out using the *umu* test (Oda et al., 1985). The system consisted of 0–100 μg/ml of test com-

pounds, 2.5 ml of exponentially growing cells (OD_{600} adjusted to 0.25–0.3) and 33 mM phosphate buffer, pH 7.4. The mixture was incubated at 37°C for 180 min and was centrifuged at 3020 $\times g$ for 10 min. The pellet was washed 3 times with 0.1 M phosphate buffer, pH 7.4, resuspended in 3 ml of the same buffer and OD_{600} was determined. 0.2 ml of this suspension was added to 1.8 ml of Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl and 1 mM $MgSO_4$, pH 7) followed by 50 μl SDS (0.1%) and 10 μl chloroform. After mixing it thoroughly, 0.2 ml ONPG solution (4 mg/ml in 0.1 M phosphate buffer, pH 7) was added to the reaction mixture and incubated at 28°C for 20 min. The reaction was stopped by the addition of 1 ml of 1 M Na_2CO_3 and the absorbances at 420 nm and 550 nm were determined. The activity of β-galactosidase was calculated according to Miller (1972). The effect of metabolic enzymes on riboflavin and lumiflavin was assessed by incorporating 0.5 ml of S9 or CCE in the original incubation mixture.

(2) SOS chromotest. The assay was performed using the procedure of Quillardet and Hofnung (1985) with minor modifications. 0.5 ml of an 18-h-old culture was diluted to 5 ml with Luria broth (1% Bacto tryptone, 0.5% yeast extract, 1% sodium chloride and 0.002% ampicillin) and incubated with shaking at 37°C for 3 h; the optical density at 600 nm was adjusted to 0.25–0.3 (2×10^5 cells/ml). The cell suspension was diluted to 10 ml with Luria broth and 0.6 ml of the diluted cell suspension was incubated with 0–100 μg/ml of test compounds at 37°C for 120 min and centrifuged at 3020 $\times$ g for 10 min. The pellet was washed 3 times with 0.1 M phosphate buffer, pH 7.4, resuspended in 0.6 ml of the same buffer and activities of alkaline phosphatase and β-galactosidase were determined. The enzyme activities were calculated according to the formula of Quillardet and Hofnung (1985). The effect of metabolic enzymes on riboflavin and lumiflavin was assessed by incorporating 5 ml of S9 or CCE to 5 ml of cells in place of Luria broth.

Ames / Salmonella assay. The liquid preincubation procedure was adopted for determining the mutagenicity of riboflavin and lumifavin (Maron and Ames, 1983). The incubation mixture consisting of 0.2 ml of a 16–18-h-old culture of tester strains, *Salmonella typhimurium* (TA100, TA98 and TA97a), 0–100 μg/ml test compounds and 0.1 ml PBS, pH 7.4, was incubated at 37°C for 30 min and centrifuged at 3020 $\times g$ for 10 min. The pellet was resuspended in 0.4 ml of 0.1 M PBS, pH 7.4. To this suspension 2 ml molten soft agar was added, mixed rapidly and spread immediately on preset minimal agar plates. The activation potential of the metabolic enzymes on riboflavin and lumiflavin was assessed by repeating the above assay in the presence of 0.1 ml S9 or CCE.

Effect of metabolic enzymes on riboflavin and lumiflavin

Aliquots of riboflavin or lumiflavin (160 μg/ml) were incubated with 3 ml S9 or CCE at 37°C for 60 min. After incubation lumiflavin was extracted with 3 ml chloroform, the solvent was evaporated off under nitrogen, the dried residue was redissolved in methanol and analysed by spectral scan and TLC. Riboflavin was extracted with amyl alcohol and quantitated by spectral scanning and TLC in the same solvent.

Protein

The protein contents of the S9 fraction and CCE were assessed using Lowry's method with bovine serum albumin as the standard (Lowry et al., 1951).

Results and discussion

Data on the mutagenicity of riboflavin and lumiflavin evaluated with the *umu* test, SOS chromotest and Ames/Salmonella assay are presented in Tables 1, 2 and 3 respectively. It was observed that riboflavin did not demonstrate any mutagenic response, before and after exposure to metabolic enzymes. These results are in agreement with the earlier reports on the non-genotoxicity of riboflavin by Purchase et al. (1978), Haveland-Smith (1981) and Combes and Haveland-Smith (1981).

In contrast to this, lumiflavin after activation by S9 and CCE showed significant mutagenicity in the *umu* test, SOS chromotest and Ames/

TABLE 1

ASSESSMENT OF GENOTOXIC POTENTIAL WITH THE *UMU* TEST

Compound (μg/ml)	β-Galactosidase activity[a] (Units/OD_{600})		
	1	2	3
0	97.00 ± 4.00	136.50 ± 4.10	137.66 ± 1.88
Riboflavin			
25	98.00 ± 1.40	144.13 ± 3.05	136.13 ± 4.07
50	101.10 ± 1.82	144.06 ± 3.61	142.80 ± 4.38
100	94.28 ± 3.30	134.23 ± 3.65	138.66 ± 5.92
Lumiflavin			
25	142.66 ± 4.26	153.31 ± 2.25	183.06 ± 5.92
50	130.20 ± 3.93	230.10 ± 6.68	236.18 ± 2.19
100	129.33 ± 7.05	267.43 ± 8.09	247.80 ± 2.76

The values represent mean ± SD of 4 independent experiments.

[a] Activity of β-galactosidase is calculated as units = $A_{420} \times 1000$/time.

1, Without S9 or CCE; 2, with S9; 3, with CCE.

Salmonella assay (Tables 1, 2 and 3). Lumiflavin per se was non-mutagenic in any of the test systems. However, it showed mutagenic response after activation by metabolic enzymes. In the presence of S9 or CCE, lumiflavin elicited a 2-fold increase in β-galactosidase activity in the *umu* test as well as in the SOS chromotest. S9-mediated activity of lumiflavin to an extent of 1.9, 2.9 and 4.8 times was also observed in *Salmonella typhimurium* TA100, TA98 and TA97a respectively in the Ames/Salmonella assay (Table 3). Lumiflavin after exposure to CCE showed mutagenic response in strain TA97a by increasing the histidine revertants to 3 times the spontaneous revertants while the other two strains remained unaffected. To our knowledge this is the first report on the genotoxic potential of a photodegradation product of riboflavin. Many compounds such as polyaromatics, aromatic amines, acridines and azo compounds are known to bring about frameshift mutations in the Ames test with and without metabolic activation (von der Hude et al., 1988). Ohta et al. have shown that the SOS function-inducing activity of 2-aminoanthracene increased markedly in the presence of S9 mix. The significance of metabolic enzymes in bringing about mutation in the Ames test is also highlighted by Prival and Mitchell (1982). Although the precise mechanism underlying lumiflavin-induced mutagenicity is not clear at present, the investigations of Kuratomi and Kobayashi (1977) with isolated DNA molecules indicated an interaction of lumiflavin with polynucleotides, especially with poly(G). They have suggested that these interactions could be ascribed to the possible intercalations of flavins with the DNA bases.

TABLE 2

ASSESSMENT OF GENOTOXIC POTENTIAL WITH THE SOS CHROMOTEST

Compound (μg/ml)	β-Galactosidase [a] units			Alkaline phosphatase [a] units			Ratio β-gal/AP			Induction factor [b]		
	1	2	3	1	2	3	1	2	3	1	2	3
0	1.49 ± 0.55	2.10 ± 0.52	1.81 ± 0.27	10.50 ± 1.00	11.00 ± 3.60	10.90 ± 1.80	0.14	0.19	0.16	1.00	1.00	1.00
Riboflavin												
25	1.66 ± 0.57	2.00 ± 0.50	2.10 ± 0.45	10.40 ± 0.50	10.30 ± 4.20	11.20 ± 2.50	0.15	0.19	0.18	1.00	1.00	1.10
50	2.10 ± 0.45	1.80 ± 0.52	1.80 ± 0.26	10.80 ± 1.30	10.10 ± 4.20	10.70 ± 3.70	0.19	0.17	0.16	1.30	0.89	1.00
100	2.20 ± 0.57	1.75 ± 0.25	1.60 ± 0.23	10.70 ± 2.8	10.70 ± 1.40	11.20 ± 2.40	0.20	0.16	0.14	1.40	0.84	0.87
Lumiflavin												
25	1.56 ± 0.40	3.67 ± 0.58	3.90 ± 0.36	11.40 ± 4.40	10.90 ± 2.80	10.30 ± 0.98	0.14	0.33	0.28	1.00	1.73	1.75
50	1.60 ± 0.36	4.00 ± 1.00	3.26 ± 0.25	10.60 ± 0.65	10.40 ± 1.60	10.50 ± 0.50	0.15	0.38	0.31	1.00	2.00	1.93
100	1.80 ± 0.34	4.20 ± 0.26	3.60 ± 0.36	11.30 ± 2.20	10.40 ± 1.20	11.00 ± 0.85	0.15	0.40	0.32	1.00	2.10	2.00

Values represent mean ± SD of 4 independent experiments.

[a] Activities of β-galactosidase and alkaline phosphatase are calculated as units = $A_{420} \times 1000$/time.

[b] Induction factor = R at tested concentration/R at concentration zero.

1, Without S9 or CCE; 2, with S9; 3, with CCE.

TABLE 3

ASSESSMENT OF GENOTOXIC POTENTIAL WITH THE AMES/SALMONELLA ASSAY

Compound (μg/ml)	Number of revertants								
	TA100			TA98			TA97a		
	1	2	3	1	2	3	1	2	3
0	104.40 ± 15.60	85.66 ± 21.30	68.30 ± 13.50	26.22 ± 4.10	21.80 ± 5.30	23.00 ± 5.70	105.30 ± 12.41	120.10 ± 31.19	119.20 ± 20.80
Riboflavin									
25	73.37 ± 12.80	81.00 ± 22.50	78.16 ± 32.50	18.25 ± 6.60	25.40 ± 3.90	19.50 ± 5.50	101.00 ± 10.80	99.12 ± 12.50	105.12 ± 19.70
50	79.70 ± 17.10	77.12 ± 19.85	72.00 ± 23.50	17.25 ± 4.10	21.66 ± 7.90	23.20 ± 3.80	111.50 ± 28.40	143.60 ± 27.40	126.80 ± 24.70
100	85.50 ± 16.80	67.40 ± 23.19	87.80 ± 14.30	23.00 ± 7.30	22.50 ± 5.0	24.10 ± 3.90	88.00 ± 5.70	127.25 ± 15.40	106.80 ± 10.22
Lumiflavin									
25	95.25 ± 32.20	139.10 ± 33.20	71.50 ± 0.70	28.60 ± 3.70	35.10 ± 4.50	25.40 ± 3.80	186.00 ± 20.21	197.80 ± 29.73	280.40 ± 46.70
50	92.33 ± 12.61	146.33 ± 24.33	97.60 ± 19.90	20.85 ± 4.37	48.33 ± 7.80	24.00 ± 3.39	177.10 ± 28.41	488.18 ± 108.70	295.40 ± 61.80
100	60.28 ± 12.20	163.37 ± 26.40	66.60 ± 15.20	25.50 ± 5.80	63.40 ± 12.80	22.40 ± 2.30	172.90 ± 16.20	583.12 ± 69.90	360.00 ± 49.50

Values represent mean ± SD of 6 independent experiments.
1, Without S9 or CCE; 2, with S9; 3, with CCE.

TABLE 4

TLC OF RIBOFLAVIN AND LUMIFLAVIN AFTER EXPOSURE TO THE MAMMALIAN METABOLIC ENZYMES S9 OR CCE

Sample	Rf
Riboflavin	0.71
Lumiflavin	0.63
Rb + S9	0.71
Rb + CCE	0.72
Lm + S9	0.62
Lm + CCE	0.63

Riboflavin and lumiflavin were separated by TLC using the solvent system pyridine–glacial acetic acid–water (20:2:80, v/v).

Such intercalations can lead to alteration in the reading frame of DNA culminating in frameshift mutations (Auerbach, 1976). Our results do indicate a positive mutagenic response (Table 3) in tester strains TA98 and TA97a which detect frameshift mutations. Observation with tester strain TA100 points to yet another mode of DNA damage by lumiflavin, namely base-pair substitution. Different mechanisms such as replacement of bases can lead to base-pair substitution (Stanier, 1976).

The treatment with the metabolic enzymes S9 and CCE did not alter Rf values of riboflavin (0.72) and lumiflavin (0.63) (Table 4). No spectral change was observed with riboflavin, before or after the treatment with metabolic enzymes (Fig. 1). The absorption maximum of lumiflavin at 221 nm, however, was found to shift to 232 nm (Fig. 2) and to 229 nm respectively on exposure to S9 and CCE (Fig. 3).

It is interesting to note that lumiflavin expresses mutagenicity only after its activation by metabolic enzymes. Enzymes of S9 and CCE are known to activate a variety of chemicals by epoxidation, oxidation, reduction, hydroxylation, acetylation and by conjugation reactions (Bartsch et al., 1982). The near identical spectral characteristics (Fig. 3) and Rf values (Table 4) observed for riboflavin and lumiflavin before and after the treatment with metabolic enzymes suggest that these compounds did not undergo major structural alterations during activation. However, the

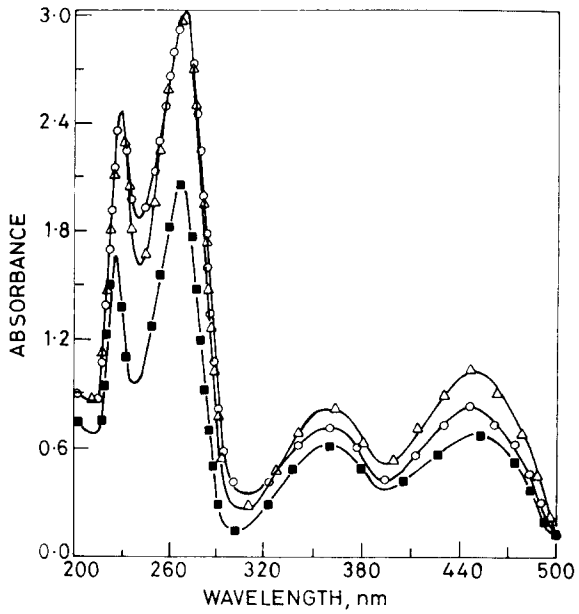

Fig. 1. Effect of S9 and CCE on the spectral characteristics of riboflavin. ■, Riboflavin; △, riboflavin + S9; ○, riboflavin + CCE. Details regarding the treatment of riboflavin with S9 and CCE and the extraction of the products are given in Materials and methods.

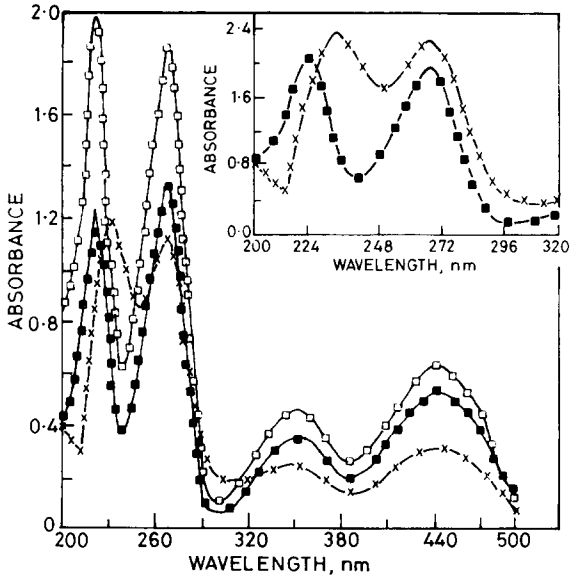

Fig. 2. Effect of S9 on the spectral characteristics of lumiflavin. ■, Chloroform extract of lumiflavin; □, standard lumiflavin; ×, lumiflavin + S9. Details regarding the treatment of lumiflavin with S9 and the extraction of the products are given in Materials and methods. The spectral pattern at 200–320 nm is expanded in the inset.

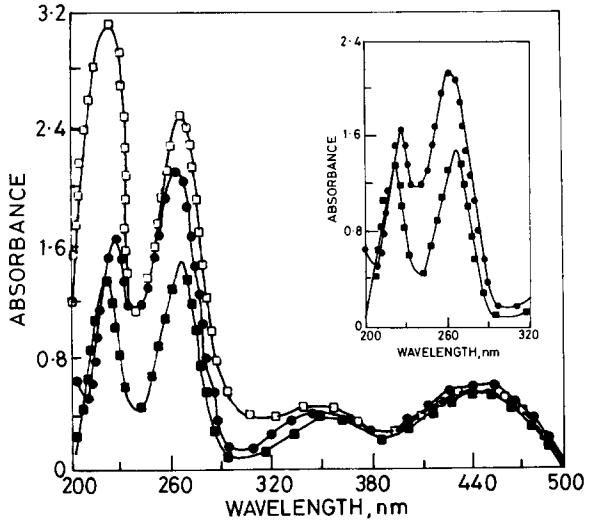

Fig. 3. Effect of CCE on the spectral characteristics of lumiflavin. ■, Chloroform extract of lumiflavin; □, standard lumiflavin; ●, lumiflavin + CCE. Details regarding the treatment of lumiflavin with CCE and the extraction of the products are given in Materials and methods. The spectral pattern at 200–320 nm is expanded in the inset.

minor (10 nm) shift of peak in the case of lumiflavin indicated that the changes may not be extensive (Figs. 2, 3 and 4). Other modifications such as tautomerisation or the production of reactive oxygen species which has the potential to induce mutagenicity may also be involved (Winston and Cederbaum, 1983; Albano et al., 1988).

The foregoing discussion clearly indicated that lumiflavin, a photodegradation product of riboflavin, can induce mutagenicity. This is particularly significant because many naturally occurring food products contain riboflavin which has the potential to produce lumiflavin-like compounds (Holmstrom, 1964). Findings in our own laboratory have shown that exposure of riboflavin to sunlight in aqueous model systems as well as in natural food products like milk can lead to its conversion to form lumiflavin.

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Technical Note

Separation, Identification and Quantification of Riboflavin and its Photoproducts in Blood Products using High-performance Liquid Chromatography with Fluorescence Detection: A Method to Support Pathogen Reduction Technology¶

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ABSTRACT

A medical device using riboflavin (RB) and light is being developed for the reduction of pathogens in platelet concentrates (MIRASOL™ pathogen reduction technology [PRT]). A high-performance liquid chromatography (HPLC) method for the quantification of RB and its main photoproduct, lumichrome (LC) in blood components has been developed and validated. In addition, the same method has been used to identify and quantify the presence of additional photoproducts–catabolites of RB. Levels of these agents before and after treatment as well as endogenous levels present in normal donor blood are reported using this analytical technique. The method allows for quantitative and qualitative analysis of RB and LC in blood components using HPLC-fluorescence detection, a Zorbax® SB-CN (stable bond cyano) column and a methanol–water mobile phase. Quantitation and qualitative analysis of additional photoproducts of RB was also performed, but the method has not been validated for these other components. The method described has passed an 8 day validation and has been found to be adequate for its intended use. The range of the method for RB is 0.016–1.500 μ*M* and for LC is 0.060–1.500 μ*M*. The method detection limit for RB is 0.0006 μ*M* and for LC is 0.012 μ*M*. The acceptance criteria for repeatability were met; the relative standard deviation for RB was 0.64% and for LC was 0.76%. The acceptance criteria for bias were met with a 97% average recovery for RB and a 102% recovery for LC. Samples were centrifuged and diluted 1:50 with 0.9% saline before analysis. No protein precipitation or extraction was required. A mass balance of approximately 93.4–94.4% was achieved after exposure of products to UV light in the intended pathogen reduction treatment method. The method permitted the identification of photoproducts in blood that were both naturally occurring and produced after photolysis of blood samples treated with the PRT process. The identity of these photoproducts has been established using HPLC Tandem Mass Spectrometry (MS/MS) and UV spectroscopic methods and has been correlated with known metabolites and catabolites of RB. HPLC with fluorescence detection using a reverse phase cyano-column allows for accurate separation, identification and quantification of both RB and LC in blood products without the need for solvent extraction or protein precipitation. Additional photoproducts could also be identified and quantified using this method. The presence of these agents in normal, untreated blood suggests that their presence in blood is ubiquitous.

INTRODUCTION

The collection, separation and transfusion of red blood cells, platelets, whole plasma and fractionated plasma components are mainstays of our health care system. Each of these elements is essential for the preservation of life and the treatment of disease. Despite years of effort, suitable substitutes have yet to be developed.

In the early 1980s, it became clear to the medical community that these life-sustaining and essential therapeutic elements were transmitting life-threatening diseases (1). The primary causative agents of these diseases were identified as the human immunodeficiency virus (HIV) and hepatitis C virus. Because of the delay in development of suitable detection systems, these agents passed undetected into the blood supply. The fact that these potentially fatal diseases were transmitted by blood, and the lack of suitable blood substitutes, posed a tremendous challenge for members of the medical community. The resulting concerns

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Abbreviations: FAD, flavin adenine dinucleotide; FMF, formylmethyl flavin; FMN, flavin mononucleotide; HIV, human immunodeficiency virus; ^{1}H NMR, proton nuclear magnetic resonance; HPLC, high-performance liquid chromatography; HPLC-MS/MS, high-performance liquid chromatography-Tandem Mass Spectrometry 2KF, 2′ keto-flavin; 4KF, 4′ keto-flavin; LC, lumichrome; PRT, pathogen reduction technology; RB, riboflavin; TCA, trichloroacetic acid; UV-VIS, UV–visible.

raised by these events triggered a surge of research into methods to purify, reduce or eliminate infectious agents in the world's blood supply.

We are developing a medical device for the reduction of pathogens in platelet concentrates. The technology uses light and the photosensitizer riboflavin (RB) (vitamin B_2). The extent of our interest in RB as a pathogen reduction agent for blood stems from over 70 years of research literature that details vitamin B_2's chemistry, toxicology and the *in vitro* and *in vivo* function in sensitizing the photochemistry of nucleic acids (2–4). Interest in the chemistry of this compound was based on its involvement in metabolic and nutritional functions as well as its behavior in individuals subjected to phototherapy (5–8). Before this research, RB had never been evaluated as a possible sensitizer for use in the *ex vivo* photochemical treatment of blood products. The presence of RB in our diets (9–11), the extensive knowledge of its photochemical properties and its well-behaved toxicological profile makes it an ideal candidate for a blood additive in this application (12,13). Previous studies have demonstrated the ability of this process to inactivate viruses including Human Immunodeficiency Virus (HIV), Porcine Parvovirus (PPV) and West Nile Virus (WNV) and bacteria including *S. epidermidis* and *E. coli* in platelet products while maintaining normal cell quality parameters suitable for transfusion (14).

One of the concerns about using any chemical agent as an additive to blood components for use in pathogen reduction technology (PRT) applications arises from the potential introduction of new chemical agents into the blood supply. These chemical agents may, on their own accord, raise concerns regarding increased toxicological risks that could, in certain circumstances, outweigh the risks associated with potential disease transmission of the blood component. The toxicological profile of RB is very well known and characterized. However, after exposure of RB to light, photochemical reactions can lead to the degradation of the molecule, yielding several photoproducts (15). These photoproducts result primarily from the decomposition of the ribityl side chain in the parent molecule (16,17). Several of these intermediates and breakdown products have been isolated and characterized (16–19).

Exposure of RB in aqueous solution to light leads to rapid photobleaching, measured at the absorption maximum of 447 nm (20). At alkaline pH, lumiflavin is a major breakdown product of RB (20). Under neutral and acidic conditions (the pH at which the pathogen reduction process is performed), lumichrome (LC) is the main breakdown product. Several intermediate by-products produced through metabolic or photochemical degradation of RB have also been identified. These include the 2′ keto-flavin (2KF) and 4′ keto-flavin (4KF) and formylmethyl flavin (FMF) (16). The isolation and characterization of these agents has been hindered by both the low levels present in isolated samples and the low sensitivity of methods of isolation and quantification available to the research community for study of these agents.

The ability to identify these agents in blood components as they naturally occur and the further ability to quantify or characterize them in samples before and after PRT treatment would permit direct examination of the potential impact of PRT on blood component chemistry and toxicology. Such studies, although not definitive, could be used to examine potential consequences in this regard in the blood transfusion setting. Hence, in support of clinical trials and toxicology studies involving this treatment regimen, we have developed a novel high-performance liquid chromatography (HPLC) method for the separation, identification and quantification of RB, LC and other photoproducts of RB in apheresis platelets both before and after PRT treatment. Typically, the measurement of flavins in blood components involves trichloroacetic acid (TCA) precipitation of proteins (21–34). This evolved from the earlier Warburg and Christian methods and was used with high salt (usually ammonium sulfate) both to dissociate flavocoenzymes (flavin mononucleotide [FMN] and flavin adenine dinucleotide [FAD]) from flavoenzymes and more recently to inactivate enzymes that could hydrolyze the flavocoenzymes or even catabolize the released RB. Subsequent extraction and concentration of the flavins was done with phenol or benzyl alcohol.

In this study we describe a sensitive and robust method for the accurate determination of both RB and LC in blood components without the need for protein precipitation or extraction. We also discuss the findings from direct examination of blood components before and after addition of RB and exposure to light as is proposed in the blood sterilization treatment protocol. These results are presented in terms of potential toxicological and medical implications for this blood safety initiative.

Table 1. Solvent gradient program parameters for HPLC method

Time (min)	Methanol (%)	Water (%)
0.0	23.0	77.0
8.0	27.8	72.2
16.0	60.0	40.0
17.0	100.0	0.0
18.0	100.0	0.0
19.0	23.0	77.0
25.0	23.0	77.0

MATERIALS AND METHODS

Chemicals. HPLC grade methanol was purchased from Fisher Scientific (Pittsburgh, PA). RB and LC were purchased from Sigma–Aldrich (St. Louis, MO). RB was used without further purification. LC was purified as described below. Reagent grade water was prepared using a Barnstead E-Pure water purification unit (Dubuque, IA). Saline (0.9%) was purchased from B. Braun Medical Inc. (Irvine, CA).

HPLC apparatus and chromatographic conditions. The HPLC system consisted of an Agilent 1100 equipped with a quaternary pump (model G1311A), an autosampler (model G1329A), an autosampler thermostat (model G1329A), a thermostatted column compartment (model G1316A), a fluorescence detector (model G1314A) and a fraction collector (model G1364A). The fluorescence detector flow cell volume was 8 μL. The HPLC column used was a Zorbax® 80Å SB-CN, 4.6 × 250 mm, 5 micron. The precolumn was a Zorbax® 80Å SB-CN 4.6 × 12.5 mm, 5 micron. The nominal backpressure was 96 bar at the beginning of the run. The fluorescence detector settings were changed during each run. Initial fluorescence detector settings were maximized for detection of RB followed by maximum sensitivity for LC. The settings were: excitation 268 nm, emission 525 nm for the first 14.5 min, followed by excitation 260 nm, emission 470 nm. The small change in baseline upon wavelength change did not significantly affect the integration. A manual injector program was set up using four wash vials (reagent grade water) to wash the needle between runs and prevent carryover of RB and LC into the next run. The left- and right-column temperatures were set to 50°C. The autosampler temperature was set to 5°C. Flow rate was 1.0 mL/min. The injection volume was 10 μL. Run time was 25 min.

Reagent grade water and methanol were used as the mobile phase and a gradient program was set up (Table 1) to separate RB and LC from the other photoproducts. A six-point calibration curve was used with RB and LC concentrations ranging from 250 to 1500 n*M*. Calibration stock

consisted of 50 μ*M* RB and 50 μ*M* LC in 0.9% saline, pH 5.0. All stock sample concentrations were confirmed by spectrophotometric analysis of RB and LC determined from extinction coefficients of the purified compounds. The stock was diluted to the appropriate calibration concentrations with 0.9% saline. An excellent straight-line fit was obtained for both RB and LC ($r^2 \geq 0.999$ in both cases). The precolumn was replaced after every 30–60 samples.

HPLC-MS/MS analysis. Photoproducts of RB were isolated in 10 separate fractions using a fraction collector. Injection volume was maintained at 500 mL. A diode array detector was used during the separation with settings of stored range from 190 to 500 nm, peak width <0.05 min, slit 4 nm.

The following table contains the fraction collection start and stop times:

Fraction number	Start time (min)	End time (min)	Duration (min)
1	3.9	4.3	0.4
2	4.4	4.8	0.4
3	5.2	5.5	0.3
4	5.6	6.0	0.4
5	6.1	6.4	0.3
6	6.6	7.0	0.4
7	8.9	9.3	0.4
8	9.7	10.0	0.3
9	11.6	12.0	0.4
10	13.2	13.4	0.2

Isolated fractions were analyzed using the diode array detector to identify UV maxima in the individual peaks. Samples were then forwarded to Cardinal Health (Raleigh, NC) for HPLC-MS/MS analysis. They were protected from light and shipped at room temperature. For analysis, samples were transferred to dark glass vials at room temperature. Analysis of all samples was performed using a Cohesive Technologies 2300 HPLC system coupled to a Sciex API3000 triple quadrupole mass spectrometer. Extractions of each of the unknown solutions were performed on a Cohesive Cyclone-P 1 × 50 mm extraction column and on either a 250 × 4.6 mm cyano or a 150 × 4.6 mm cyano analytical column for analytical separations. All mass spectrometric analysis was done using electrospray negative ionization. In cases where more signal was needed, samples were concentrated using a Turbo-vap at 30°C with nitrogen gas. The instrument was protected from light during the evaporation. For all injections, the following MS/MS transitions were scanned, based on analysis of standards: RB, 375.1 > 255.1 amu; KF, 373.1 > 241.3 amu; LC, 240.9 > 198.1 amu; FMF, 282.9 > 240.2 amu.

Apheresis platelet preparation. Single donor platelets (a minimum of 270 mL) were collected by an accredited blood bank facility using a Gambro BCT Inc. TRIMA® Automated Blood Component Collection System. The platelet product was held between 2 and 30 h in the TRIMA collection bag before subsequent processing. The bag containing the platelets was connected to the pathogen reduction illumination–storage bag (ELP™) using a Terumo® Sterile Tubing Welder. After sterile connection, 250 ± 5 mL of platelet product was transferred into the illuminator bag, which contained 28 mL of RB solution (500 μ*M*). The transfer tubing was sealed using a Sebra® hand-held, radio frequency tubing sealer. After connection, the two bags were separated and the original collection bag was discarded. Each final platelet product to be treated contained 1260×10^3 to 1690×10^3 platelets/μL suspended in approximately 278 mL of 90% autologous plasma in a 1 liter citrate-plasticized, polyvinyl chloride ELP bag with an illumination surface area of 347 cm^2/side.

Plasma derived from buffy coats. Buffy coat platelets were prepared from whole blood that was stored at room temperature for 16–24 h after collection. The platelets were spun down at 16 100 RCF for 3 min and diluted 1:50 with 0.9% saline before analysis.

The PRT process. A complete description of the procedures used for this process has been described previously (Ruane *et al*.). Briefly, the platelet concentrates were treated separately with 6.2 J/mL of light. The lamp phosphor possesses an output range from 265 to 370 nm. The platelet product was placed in a product chamber where mixing (on a motorized platen) and exposure to light took place. Total illumination ranged from 8 to 10 min. Dedicated fans were used to cool each lamp chamber and product chamber. Product temperatures during illumination ranged from 22°C to 24°C.

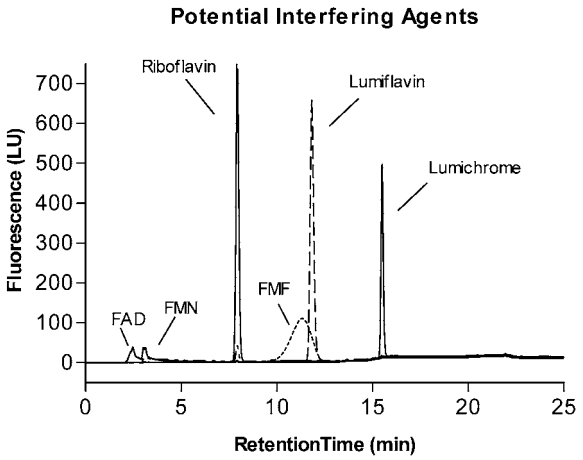

Figure 1. HPLC chromatograms of samples containing various potential interfering agents including lumiflavin, FMN, FMF and FAD.

Purification of LC. The average purity of reagent LC (Aldrich PN 10,321-7) was found to be approximately 68–85% as determined by negative ion mass spectroscopy and HPLC. LC was purified before use as an analytical standard as follows: reagent grade LC (250 mg) was added to a 250 mL amber volumetric flask and diluted to the mark with reagent grade water. The solution was sonicated (VWR® sonicator model 150HT) at 80°C for 2 h and then vacuum filtered while hot, using a 0.45 μm filter. The filter cake, which consisted of purified LC, was dried at 105°C for 2 h (the impurity is soluble in water and LC is relatively insoluble). The LC purity after this process was found to be greater than 99%. LC purity was confirmed using mass spectrometry, proton nuclear magnetic resonance (^{1}H NMR), HPLC and elemental analysis. An API3000 triple quadrupole mass spectrometer using negative ionization mode observed the parent M–1 ion 241 *m/z* concurrent with the LC nominal molecular weight of 242 g/mol in the purified sample. Elemental analysis—found: C 59.04, H 3.81, N 22.95; required: C 59.50, H 4.16, N 23.13. ^{1}H NMR (D_2O), 2.39, s, 3H; 2.41, s, 3H; 7.61, s, 1H; 7.78, s, 1H.

Preparation of FMF reference compound. FMF was synthesized according to the method of Fall and Petering and characterized using NMR and mass spectrometry (35).

Calibration standard preparation. Analytical standards were prepared by diluting a stock solution of 50 μ*M* RB and 50 μ*M* LC in saline. The stock solution was prepared by adding LC (12.1 mg) to a 1 liter amber volumetric flask and adding approximately 950 mL of pH 5.0 saline. The solution was sonicated using a VWR sonicator (model 150HT) at 70–100°C for approximately 2 h until all LC was in solution. RB (18.8 mg) was then added and the solution allowed to cool while stirring overnight. It was then filled to the mark with pH 5.0 saline. Calibration standards were prepared fresh every 24 h.

HPLC validation for quantitative analysis of RB and LC. Validation of the method for RB and LC, the principal photoproduct of RB was carried out during an 8 day period. Both these agents were present in sufficient quantities in blood products to validate the methodology. Other components (FMF, 2KF, 4KF) were isolated by bulk HPLC and characterized by mass spectroscopy and UV–visible (UV-VIS) spectroscopy. Results from these studies were used to determine concentrations of these agents in blood components, but validation of the methodology for quantitative determination of these components was not performed. Linearity of the calibration curve was 0.999 (r^2) or better for all 8 days of validation. For five replicates of both RB and LC at three concentrations (25, 37.5 and 50 μ*M*), the average relative standard deviations were RB = 0.64% and LC = 0.76%. For these 30 samples, the average percent recovery for RB was 97% whereas the average percent recovery for LC was 102%. The limit of quantitation for RB (undiluted) was 16 n*M* and for LC was 60 n*M*. Samples were diluted 1:50 with 0.9% saline pH 5.0 before analysis. The range of the method for RB is 0.016–1.500 μ*M* and for LC 0.06–1.500 μ*M*. A typical analysis involved two replicates of each sample. No loss of peak shape, peak height or retention time was noted for runs using as many as 15 samples (30 samples when replicates are accounted for).

During the 8 day validation, all retention times for RB samples were within 7.8 ± 0.2 min and all retention times for LC samples were within

Table 2. RB and photoproducts characteristics

Compound	Average HPLC retention time (min)	UV_{max} (nm)	MS/MS transitions (amu)
RB	8.4	223, 267, 374, 444	375.1 > 255.1
LC	15.5	218, 260, 352	240.9 > 198.1
2KF	9.1	224, 268, 372	373.1 > 241.3
		446	373.1 > 255.1
4KF	9.9	222, 268, 374	373.1 > 241.3
		446	373.1 > 255.1
FMF	10.8	222, 268, 372, 446	282.9 > 240.2

15.4 ± 0.2 min. The average resolution was 10.1 and the average tailing was 0.34, passing the Food and Drug Administration validation requirements of ≥2.0 resolution and ≤2.0 tailing (36).

Reinjection precision analysis was performed to determine whether an analytical run could be restarted in case of instrument failure. Analysis was performed on a calibration curve and on 30 samples, 10 each at three different concentrations. The calibration curve was followed by 30 samples, including blanks and check standards every 10 samples, simulating a routine analysis. After completion of the sequence, the entire sequence was restarted. The difference in measured RB and LC concentrations from the first run and second run was less than ±1.5%.

Interference. Several analytes were run to test for interference including FAD, FMN, lumiflavin and FMF. None of these analytes interfered with the analysis of RB and LC. Results from sample runs with these agents are included in Fig. 1.

RB and photoproduct analysis in platelet concentrates. Ten platelet concentrates spiked with 50 μ*M* RB were treated with 6.2 J/mL light. The concentrations of RB, LC and other photoproducts were measured before and after spiking with RB, after illumination and after 5 days of storage on a Helmer shaker, respectively (22–24°C, shaker speed of 72 ± 5 cpm). All samples were stored in the dark until analysis, whereupon a 4 mL sample was drawn from each platelet product. Each sample was centrifuged at 3000 RCF for 10 min to remove the cellular components. The supernatant was respun at 16 100 RCF for 3 min and the supernatant diluted 1:50 with 0.9% saline. The samples were then placed in amber HPLC vials for analysis. Analysis included characterization of peaks associated with the 2KF, 4KF and FMF as well. Quantification of these latter samples was conducted on the basis of extinction coefficients for the parent molecule and HPLC analysis. Identification of peaks associated with these agents was performed through analysis of isolated fractions by mass spectroscopy–HPLC and UV-VIS absorption characteristics (Table 2). In the case of FMF, direct comparison with a *de novo* synthesized standard was possible (Table 3).

Additional samples taken from normal donors were analyzed for the presence of RB and RB catabolites or photoproducts. These samples were assayed directly without any RB addition to determine the levels of each component present in a natural state in blood components.

RESULTS

RB, LC, 2KF, 4KF and FMF analysis in apheresis platelets

The level of RB and LC and other catabolites of RB in the platelet products after spiking with 50 μ*M* RB are listed in Table 4. The average RB concentration after the addition of 28 mL of 500 μ*M* RB to 250 ± 5 mL platelet product was 48.3 μ*M* (n = 10, Table 4). PRT treatment resulted in 78.9% (n = 6) recovery of the RB originally present after dilution and before illumination. An additional 6.2% of this initial level of RB was recovered in the form of LC, resulting in a total of 85.1% recovery of the initial level of RB. The remaining levels were identified to be composed of FMF, 2KF and 4KF. Separation of these components by bulk HPLC was conducted to isolate purified fractions of each. These samples were examined to obtain mass spectra data and UV-VIS spectra characteristics. Upon storage for 5 days, the amounts of RB and LC both increased by a measurable extent (Fig. 2b,c). A total of 80.8% RB (n = 10), 7.4% LC, 1.4% 2KF, 0.3% 4KF and 3.5% FMF were recovered (n = 10), yielding a total mass balance of 94.4% after 5 days of storage. These results are given in Table 4. The HPLC chromatograms of the PRT-treated platelets before illumination, after illumination and after 5 days of storage are shown in Fig. 2.

Table 3. FMF standard *versus* isolated compound

Compound (FMF)	Retention time (min)	UV_{max}	Mass spectra ions(*m/z*)
Standard reference	10.8	224, 268, 372, 446	283 (parent), 240 (daughter)
Isolated compound	10.8	222, 268, 372, 446	283 (parent), 240 (daughter)

Quantitative analysis of endogenous RB and photoproducts–catabolites in plasma

A modified version of the current method was used to measure endogenous concentrations of RB and RB catabolites in plasma. The injection volume was increased from 10 to 100 μL and the dilution with saline was reduced from 1:50 to 1:5. This gave a 100-fold increase in RB detection limits. A six-point calibration curve was run on the same day of analysis using standards ranging from 1 to 50 n*M* (1, 2, 5, 10, 20 and 50 n*M*). Thirty samples of plasma derived from buffy coat platelets were analyzed for RB and its photoproducts–catabolites. The average concentration of RB was 23.9 n*M* (range 8.6–79.6 n*M*) (see Fig. 3). Excellent results were obtained with this adapted HPLC method. Blanks were run before and after the calibration curve and between each of the 30 samples. RB was not detected in any of the blanks. The linear regression of the calibration curve was greater than 0.999. Levels of LC and other photoproducts–catabolites of RB present in these products varied as shown in Table 5. Out of 30 products in total analyzed using this method, all samples demonstrated measurable levels of RB. Only 23 of the 30 products demonstrated measurable levels of LC. A total of three of the 30 had measurable levels of all four major photoproducts. These also corresponded to those samples having the highest concentrations of RB. The average concentrations determined for each of these products as well as the ranges observed are listed in Table 5.

DISCUSSION

The methodology described represents a sensitive HPLC method for the quantification of RB, LC, 2KF, 4KF and FMF in blood components that does not require a protein precipitation or organic solvent extraction step. The method simply removes the cellular components by centrifugation followed by dilution with saline and analysis using a reverse phase cyano-column (Zorbax® 80Å SB-CN, 4.6 × 250 mm, 5 micron). We have found that the low concentration of protein after dilution does not interfere with the assay.

Typically, C18 reversed phase columns are used to analyze RB and related compounds in aqueous solutions (15,18–20,22,24–29,31–34, 36). However, we found that the use of this stationary phase was not satisfactory and separation of photoproducts from RB after photolysis was not feasible. A reverse phase cyano-column was found to be optimal in resolving compounds closely eluting to RB (Fig. 2). Other columns were used and separation was not optimal

Table 4. RB and photoproducts in apheresis platelets*

	PRE (μ*M*)†	POST (μ*M*)‡	POST 5 days (μ*M*)†
RB	48.33 ± 1.13 (47.1–50.4)	38.68 ± 0.68 (38.0–39.9)	39.65 ± 1.22 (37.0–41.3)
% of initial		78.9	82.0
LC	0.23 ± 0.08 (0.1–0.3)	2.90 ± 0.20 (2.5–3.0)	3.60 ± 0.30 (3.2–4.1)
% of initial		6.2	7.5
2KF	N/A	1.27 ± 0.17 (1.10–1.49)	0.71 ± 0.12 (0.59–0.90)
% of initial		2.57	1.44
4KF	N/A	0.53 ± 0.03 (0.48–0.58)	0.16 ± 0.04 (0.11–0.22)
% of initial		1.06%	0.32%
FMF	N/A	1.99 ± 0.24 (1.63–2.32)	1.72 ± 0.22 (1.42–2.07)
% of initial		4.02	3.49
Totals		45.4 μ*M*; 93.4%	45.8 μ*M*; 94.4%

*PLT, apheresis platelet product before spiking with 50 μ*M* RB; PRE, after spiking (50 μ*M* RB); POST, after treatment; POST 5 days, after a further 5 days of storage.
†n = 10.
‡n = 6. Values in parentheses are the range.

(Agilent (Zorbax) Columns: SB-C18, XDB-C18, 300 Extend C18, 300 SB-C8, XDB-C8, SB-C8, 300SB-C3, 300SB-CN, Carbohydrate, SB-Aq, Eclipse AAA, NH2, Diol and GF-250).

The measurement of vitamin B_2 in blood components is not new (15,16,19–24,32). Typically, FAD in the blood is converted to the more stable FMN. Free RB and FMN are then measured after their extraction into organic solvents. These methods also require the acidic (usually TCA) precipitation of blood proteins before analysis. This has one significant drawback. RB and related compounds tend to coprecipitate with the protein making accurate determinations difficult. In most cases, although agents such as FMF, 2KF and 4KF have been previously identified, their low levels in naturally occurring blood products and the low sensitivity inherent in previous methodologies prohibited their routine identification and quantification in blood components. The increased sensitivity of the method described in this study permits this direct analysis.

The method described in this article has been developed to support a PRT known as MIRASOL PRT. The technology involves the inactivation of pathogens in platelet concentrates using 50 μ*M* RB and light. During the course of this study, we measured the amount of RB, LC, FMF, 2KF and 4KF in 10 apheresis platelet products before and after treatment. The platelet concentrates were also stored at 22–24°C on a Helmer shaker for 5 days, whereupon the amounts of each of these agents were reanalyzed. We were able to accurately determine the amount of RB converted to photoproducts during the process. A total of 78.9% RB remained immediately after irradiation. This amount increased upon 5 days of storage to 82.0%, presumably because of photoproducts (not LC) reverting back to RB during this period. The amount of LC also increased upon storage from 6.2% to 7.5%. The total mass balance of both compounds on Day 5 was 89.5%. The remaining 10.5% is made up of other photoproducts that have been positively identified and quantified through mass spectroscopic and other analytical methods. Figure 2 shows an overlay of HPLC chromatograms of RB in a diluted platelet product (1:50), before illumination (a), after illumination (b) with the PRT process and Day 5 after treatment (c). The photoproducts β and γ have been identified as 2KF and 4KF, respectively. Their UV-VIS absorbance spectra (both are nearly identical to that of RB) and mass spectra are consistent with this hypothesis. Results from HPLC, UV-VIS analysis and mass spectra data are depicted in Table 2. FMF (δ) has been positively identified by comparison with an authentic sample. Results from this analysis are depicted in Table 3. Quantification of these photoproducts has been conducted using the extinction coefficients for the parent molecule and are included in Table 4.

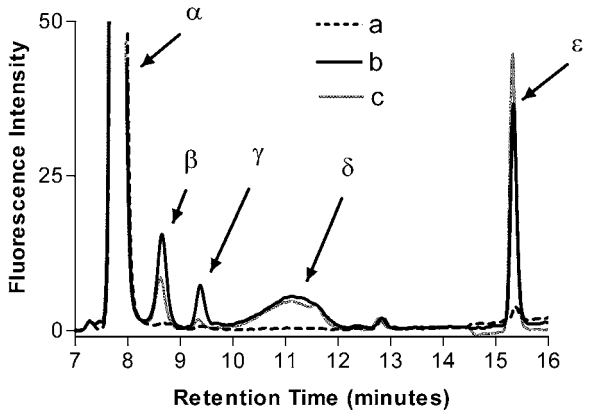

Figure 2. RB (50 μ*M*, diluted 1:50 with 0.9% saline, pH 5.0) in apheresis platelets illuminated 6.2 J/mL with UV. (a) Before illumination. (b) After illumination (RB = 78.9%, LC = 6.2%). (c) After illumination and 5 days storage (RB = 82.0%, LC = 7.5%). α, RB; β, mass spectrum and UV-VIS are consistent with 2KF; γ, mass spectrum and UV are consistent with 4KF; δ, mass spectrum and UV are consistent with FMF; ε, LC.

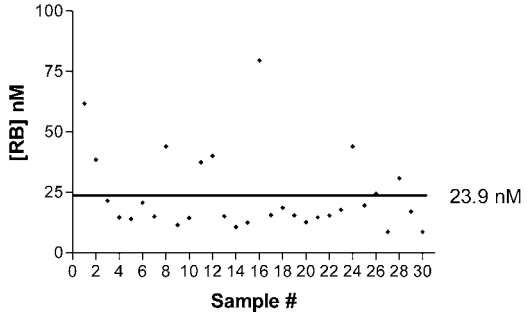

Figure 3. Endogenous concentration of RB in 30 plasma samples derived from buffy coat platelets. Average = 23.9 n*M*.