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The influence of riboflavin photochemistry on plasma coagulation factors

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Abstract

Studies with riboflavin in the 1960s showed that it could be effective at inactivating pathogens when exposed to light. The principal mode of action is through electron transfer reactions, most importantly in nucleic acids. This suggested that it could act as a photosensitizer useful in the inactivation of pathogens found in blood products.

Objective—To study the influence of photo-inactivation with riboflavin on the coagulation factors of plasma.

Methods—The photo-inactivation procedure of riboflavin plus light was applied. Fifty isogroup pools of two plasmas were made from 100 U of plasma that were derived from whole blood products that had previously been held overnight. Pools were split into two bags. One of them was photo-inactivated, and post inactivation samples were obtained. The second bag was not photo-inactivated and samples were taken. Total protein, fibrinogen, FII, FV, FVII, FVIII, FIX, FX, FXI, FXIII, antithrombin III, PC, PS, α-2 antiplasmin and vWF:Ag, the multimeric structure of vWF and ADAMTS-13 were analyzed.

Results—In plasma, the proteins most sensitive to photo-inactivation were fibrinogen, FXI, FVIII, FV, and FIX (33%, 32%, 30%, 18% and 18% loss, respectively). Coagulation inhibitors, PS, antithrombin III and PC showed little decrease (all 2%). Retention of vWF and ADAMTS-13 were 99% and 88%, respectively.

Conclusions—As with other pathogen reduction procedures for plasma products, treatment with riboflavin and UV light resulted in reduction in the activity levels of several pro-coagulant factors. Coagulation inhibitors are well preserved.

Keywords

Plasma; Coagulation factors; Pathogen reduction; Riboflavin

1. Introduction

Plasma obtained through centrifugation of whole blood or single-donor plasma contains a variety of valuable organic and inorganic elements. Delivered and stored as Fresh Frozen Plasma (FFP) it is the optimum first line therapy for many acquired coagulopathies,

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particularly those resulting in low levels of multiple coagulation proteins, when isolated concentrates are not available (e.g. factor V or XI) or for plasma exchange in Thrombotic Thrombocytopenic Purpura (TTP).

Owing to the complexity of plasma proteins and factors related to its processing and storage, FFP has the potential to cause a wide range of pathophysiological reactions, such as TRALI. FFP also contains the risk of transmission of viruses such as HIV, HBV, HCV, HAV, EBV, HHV-8, prions, and protozoa. These risks have been reduced with the introduction of careful selection procedures for blood donors, and with the implementation of screening tests for known blood borne pathogens [1], but there still looms the threat to the blood supply of a new or re-emerging pathogen [2].

It has been estimated that the residual risks from a single unit of FFP are 1 in 10 million for HIV, 1 in 50 million for HCV, and 1 in 1.2 million for HBV. Against these levels of risk, it has been questioned whether pathogen reduction in FFP is a necessary strategy and/or the best use of health-care resources [3].

Nevertheless, transfusion is still not risk-free. Transfusion- related fatalities and infections continue to be reported, and blood is currently not tested for many potentially dangerous known and unknown pathogens. The current reactive approach to blood safety, namely, adding new donor disqualifications and/or laboratory tests after each new recognized threat, has reached the limits of practicality [4]. In addition, the emergence of new agents such as West Nile Virus (WNV) and Chikungunya virus demonstrates that potential threats to the blood supply continue to emerge worldwide [5]. This reminds us that sometimes viruses move ahead of our ability to test them [3].

Two approaches have been developed so far to make FFP safer, quarantine and pathogen reduction of FFP. Quarantine FFP is an optimally prepared and stored FFP which is retested and found negative for infectious disease markers 4–6 months after collection, has good haemostatic activity, but still carries the risk of transmitting blood borne infections that are not detected by screening methods [1] and infections that are not tested for. The product has been widely used; however, the risk of emerging infections is currently challenging this practice in some countries. Furthermore, quarantine FFP shows another additional disadvantage: the logistical challenge of having to maintain a huge inventory of once-tested plasma. Pathogen reduction of blood products represents a proactive approach to blood safety, promising an additional layer of protection for known infectious agents for those that are new or not yet recognized as threats to the blood supply [2]. Pathogen reduction is the use of a process that inactivates a virus, bacteria, fungus, or protozoan pathogen from the product. The methods used should inactivate pathogens without damaging the function or shelf-life of the blood product. In addition, the products used and the resulting complexes must be demonstrated to be non-toxic and non-immunogenic [6]. All procedures developed so far to reduce pathogens in single units of plasma, use photochemical treatment [1]. Methods that are already applied for FFP intended for transfusion include the solvent/ detergent (S/D) procedure used in plasma pools, treatment with Methylene Blue (MB) and light which is suitable for single FFP units, and a pathogen reduction system to treat platelets and plasma for transfusion using amotosalen and UVA.

A new approach is available which uses riboflavin (45–85 μM) and UV light (265–370 nm) to treat platelets or plasma (MIRASOL™ Pathogen Reduction Technology System, CaridianBCT Biotechnologies, Lakewood, Colorado, USA). Riboflavin, a naturally occurring vitamin (vitamin B2), is used as a photosensitizer in combination with light, which provides energy to inactivate living micro- organisms. As a dietary nutrient, riboflavin has over 30 years of drug safety data and research work that detail the pharmacokinetics,

toxicology and function of vitamin B2 in sensitizing the photochemistry of nucleic acids. Extensive research has indicated that riboflavin and UV light inactivates viruses [7–9], bacteria [7,8], parasites [10,11], and white blood cells [12] in platelets and plasma.

Therapeutic plasma must not only be safe (i.e. free from viruses, bacteria and parasites), but to be clinically effective it must also contain sufficient levels of coagulation factors and protease inhibitors. In the current study we examined the influence of photo-inactivation with riboflavin on the coagulation factors of fresh plasma frozen within 24 h and subsequent frozen storage for at least 24 h. This study evaluated the following parameters: total protein, fibrinogen, Factors II, V, VII, VIII, IX, X, XI, XIII, Proteins C and S, antithrombin, α-2 antiplasmin, vWF and vWF multimers, and ADAMTS-13.

2. Material and methods

2.1. Collection and preparation

Whole blood units, approximately 465 mL, anticoagulated with CPD were drawn from groups A, O, B, and AB donors. Units were processed after 16 h of storage on butane-diol plates at 22 °C until plasma separation was performed within 18 h of collection. For plasma separation, a 22 ± 2 °C centrifuge hard spin was performed.

Two units of matched blood group were pooled (pools = 50: A = 18, O = 17, B = 10, and AB = 5) in the plasma pooling bag and stored on a benchtop at room temperature (22 ± 2 °C). The pooled product was split into two volumes to produce paired riboflavin (265 mL) treated and control units (the remaining volume). This was performed within 18 h from collection. The control units were placed in Universal Blood Bags (UBB) made from plasticized PVC containers suitable for plasma storage. Samples intended for riboflavin and UV light treatment were transferred to a PVC-citrate plasticized container (illumination bag).

2.2. Product illumination

The inactivation process involves the use of an illuminator that delivers UV energy to a unit of plasma in an illumination bag. Riboflavin solution was connected to the illumination bag with plasma by a sterile connector (Terumo TSCD Sterile Tubing Welder). The riboflavin solution (500 μM) bag contents (35 ± 5 mL) were drained into the illumination bag. Residual air was then gently expressed from the illumination bag into the empty riboflavin solution bag. The contents of the illumination bag were mixed manually.

The illumination bag was placed in the illuminator and exposed to UV light with a linear agitation of 120 cpm and a product temperature of < 37 °C. The bag was removed after a target energy of 6.24 J/mL was delivered (approximately 6 min), and the plasma product was transferred to a storage bag and frozen at the same time as its type matched control.

2.3. Sampling and storage preparation

The pooled plasma bag was spiked and 1.5 mL aliquots to each of 20 cryogenic 2 mL vials were transferred and labeled. These samples were held at room temperature. After illumination, using a plasma transfer set, the treated illumination bag was spiked and 1.5 mL aliquots from each unit were transferred into 2 mL cryogenic vials and labeled.

Both the pre-illumination and post-illumination cryovials were flash-frozen and stored in a −80 °C freezer for at least 24 h until analysis for protein parameters. Both samples were frozen at the same time within 24 h after the time of whole blood collection and no more

than 6 h after plasma separation. During the period of processing of the treated group, the control group was maintained at 22 ± 2 °C.

After illumination, the remaining plasma product from the illumination bag was transferred to the storage bag and labeled. The paired control and treated units were flash-frozen at the same time by means of a nitrogen ward-robe. Bags have been stored for further studies.

The plasma from the cryovials was thawed via a 37 °C water bath for analysis of the following parameters: total protein, fibrinogen, Factors II, V, VII, VIII, IX, X, XI, XIII, protein C and S, antithrombin, α-2 antiplasmin, vWF and vWF multimers, and ADAMTS-13, immediately after thaw.

2.4. Assay methods

The protein C and antithrombin determinations were done with functional assays based on a synthetic chromogenic substrate. Protein C was measured in two stages: first incubation of the plasma with protein C activator and then quantification of activated protein C with a synthetic chromogenic substrate. In the case of antithrombin, the plasma was first incubated with Factor Xa in the presence of an excess of heparin, and then the residual Factor Xa activity was quantified with a synthetic chromogenic substrate. This activity is inversely proportional to the antithrombin level in the test sample. The third chromogenic assay was the functional determination of α-2 antiplasmin.

Plasma levels of factor II, V, VII, and X were determined using functional assays based on the prothrombin time with human factor II, V, VII and X immunodepleted plasmas. Similarly, plasma levels of factor VIII, IX and XI were determined using human factor VIII, IX and XI immunodepleted plasmas, based on the partial activated thromboplastin time. A chromogenic assay was also used to determine factor VIII functional activity.

Fibrinogen was determined with the Clauss method. Free protein S was determined by measuring the increase of turbidity produced by the agglutination of two latex reagents. The first latex reagent has adsorbed C4BP which has high affinity for free protein S of patient plasma in the presence of calcium ions. The second latex reagent is sensitized with a monoclonal antibody directed against human protein S. The degree of agglutination will be directly proportional to the free protein S concentration. Factor XIII was determined with an automated latex enhanced immunoassay for the quantitative determination of factor XIII antigen. Specifically the latex particles are coated with antibodies highly specific for the active A-subunit of factor XIII. All the determinations (except chromogenic FVIII) were performed in an automated coagulation analyzer (ACL Top 3G from Instrumentation Laboratory) with HemosIL™ reagents (Instrumentation Laboratory Company, Lexington USA). The ACL Top is a fully automated, benchtop, random-access analyzer designed for in vitro coagulation testing. Total protein content was measured by a colorimetric assay (TP assay, Roche Diagnostics, IN, USA) using Roche/Hitachi Modular Analytic equipment. Chromogenic FVIII was determined using an STA Compact (Diagnostica Stago, Asnières, France) with COAMATIC factor VIII reagents (Chromogenix, Milan, Italy).

The vWF antigen level was measured with an Immuno-Turbidometric Assay utilizing a suspension of vWF-specific antibody-coated micro particles as the substrate. The vWF antigen/antibody complexes are generated after incubation with a plasma sample. The resulting agglutination of micro particles increases the turbidity of the medium and the amount of antigen can be measured photometrically. The vWF antigen can be analyzed by measuring the increase in absorbency due to micro particle agglutination, and this can be evaluated against a reference curve generated from normal control plasma.

Ristocetin co-factor activity of vWF is measured using a reagent containing human stabilized platelets. The vWF (ristocetin co-factor) causes the platelets in the substrate to agglutinate. The resulting agglutination reduces the turbidity of the medium allowing the optic density to be measured. The optic density can be measured against the reference curve constructed from normal control plasma to determine the vWF activity level.

ADAMTS-13 activity level was measured with an SDS–PAGE assay as previously described, using guanidine HCl treated vWF multimers as the substrate [13]. Proteolytic fragments generated from the substrate after incubation with a plasma sample were analyzed by SDS–PAGE and immunoblotting. The protease activity level of the sample was determined by interpolating the optical density of the dimeric 176-kD vWF fragment against a reference curve constructed from serial dilutions of normal control plasma.

3. Statistical analysis

Means and standard deviations were calculated for each of the parameters evaluated in the control group. The mean percent protein retention and standard deviation of each parameter was also calculated for riboflavin treated samples.

4. Results

The average retention of biologic activity of coagulation factors studied was 88%. Table 1 shows the mean activity of the proteins studied in the control plasma, as well as their retention in the treated plasma. In general, anti-coagulant factors were better preserved than coagulant factors.

The proteins most affected by the treatment procedure were fibrinogen (67% recovery relative to control), Factor VIII clotting assay (70% recovery relative to control), and Factor XI (68% recovery relative to control). All other factors demonstrated recovery levels relative to controls at > 80%.

Antithrombin III, proteins C and S were all maintained at 98% of the values observed in the control, untreated product pairs. A loss of high molecular weight vWF multimers was seen, but the vWF antigen showed good recovery (98%). Total protein content was fully retained in treated units.

VWF Protease activity, ADAMTS-13, following treatment was also well preserved with 88 % retention in the treated plasma.

5. Discussion

The results obtained in this study can be compared to those reported previously for other processes for pathogen reduction of plasma. This includes results with S/D, MB, and psoralen treated plasma products.

Our experience with inactivation methods started in 1998 when we began inactivating with MB all plasma intended for transfusion. Currently we are photo-inactivating 25,000 plasma units per year. Based on this experience we believe that the process we followed during this current study with riboflavin and UV light can be easily incorporated into plasma preparation steps at a blood centre. We feel that the riboflavin and UV light process is similar to our current inactivation procedures for individual plasma units using MB [1].

Most studies dealing with factors that influence plasma quality have focused on factor VIII (FVIII) activity, since this is the least stable of the plasma proteins considered here and has

been the major consideration driving the need of source plasma for fractionation. With respect to indications and clinical efficacy of therapeutic plasma, however, FVIII plasma levels are less important. FVIII is an acute phase reactant and its plasma levels are mostly normal or increased even in severe cases of complex coagulopathies. Current guidelines regarding FVIII activity in therapeutic plasma would seem therefore not to be very helpful and might well be reconsidered [14]. Virus inactivation procedures can have the consequence of reducing several clotting factors and inhibitors. In S/D treated plasma this happens to varying degrees, however, pooling of plasma units before S/D treatment results in well-standardized protein levels [15]. In the current study, the FVIII (chromogenic assay) level measured was 0.7 (0.2 IU/mL with a retention of 81%. The FVIII chromogenic assay is a more accurate measurement of samples where interference may be reduced because the chromogenic method uses a high dilution as compared to clotting methods. This dilution minimizes interference [16]. The 0.7 IU/mL with a retention of 81% reported here is comparable to the 0.8 IU/mL and 80% retention currently seen with the MB treatment at our facility.

The data obtained in this study for Factor VIII levels in treated plasma are lower than prior results reported for plasma treated with riboflavin and UV light [17]. A study where plasma was evaluated after 52 weeks (at −30 °C) showed values of Factor VIII (chromogenic) of 0.9 ± 0.2 for whole blood derived plasma [17]. In this latter study, plasma was frozen within 8 h after collection. In our current study, it was frozen within 24 h of collection. Plasma separated from whole blood donations and frozen below −18 °C within 24 h of collection (FP24) shows good retention of relevant coagulation activity [15]. However, when compared with historical data on plasma frozen within 8 h, levels of fibrinogen, Factor V, VIII and XI in FP24 in this study have been shown to be reduced by an additional 12%, 15%, 23% and 7%, respectively [17].

In this study, we also examined the effects of treatment on the anti-coagulant proteins, Proteins C and S, α-2 anti-plasmin, and anti-thrombin III. The most striking finding in SD plasma is the marked decrease in protein S and plasmin inhibitor activity levels [18–21]. Reduced protein S activity in S/D plasma may predispose to venous thrombo embolism, especially if infused in large volumes [22]. In the current study a retention of 98% was noted for protein S with mean ± SD values of 0.9 ± 0.1 IU/mL for the treated product. The levels of protein C, α-2 antiplasmin and antithrombin III were also well maintained, with > 97% retention for treated product.

In most of the studies involving single inactivated plasma units, a decrease in the levels of most coagulant factors is reported, with fibrinogen and FVIII the most affected both with MB and psoralen [23,24]. In the current study, the fibrinogen retention was 67%. There have been similar fibrinogen percent retentions reported for MB (72%) [23] and psoralen (70%) [24] treated whole blood derived plasma.

The S/D procedure does not result in a reduced vWF-cleaving protease (ADAMTS-13) activity, which is important for plasma exchange in thrombotic TTP [22,25]. However, Yarranton et al. [22] reported that S/D plasma contained reduced levels of the highest molecular weight vWF multimers with a reduced protein S activity (below the normal range). For MB treated plasma, these values remain within the normal range. A small randomized controlled trial with plasma treated with amotosalen and UVA provided encouraging preliminary evidence of effectiveness in the treatment of TTP [26]. In this study, the percent retention of vWF-cleaving protease activity was 88%.

In riboflavin treated plasma a loss of high molecular weight multimers was also seen. However, the vWF activity: antigen ratio was 1.2 ± 0.2. A ratio of > 0.7 is considered to be

normal suggesting that the vWF that is present in the plasma is functional [27]. The loss observed in this study appears to have been absent in products treated and frozen within 8 h post-collection. This may suggest that the over-night hold as whole blood, followed by separation and treatment with freezing at 24 h post-collection may have made the vWF multimers more sensitive to the riboflavin and light process. This observation warrants additional study and analysis.

We feel it is important to discuss the degree and clinical significance of factors that did change; however, it is difficult to make a conclusion regarding clinical significance as determined by in vitro protein data alone. The moderate loss of activity of some coagulation factors (15–33%) linked to the riboflavin and UV light pathogen reduction process is comparable to that reported for other inactivation procedures and these products have been deemed clinically effective in their use. The loss of activity observed for vWF and the anti-coagulant proteins (ATIII, proteins C and S, and α-2 antiplasmin) appears to be less than or comparable to what has been observed with other treatment processes that are currently available for routine clinical use in Europe.

6. Conclusions

Several pro-coagulant factors demonstrate reductions in activity from 20–30% following treatment. The quantitative and qualitative conservation of coagulation factors achieved in riboflavin-UV-light treated plasma, however, are similar to other pathogen reduction systems. Coagulation inhibitors are well preserved following treatment with this process.

After this initial experience, a comparison between a gold-standard plasma inactivation method, i.e. MB, and riboflavin and UV light is warranted.

Further randomised, prospective, clinical and pharmacovigilance studies will be necessary to show how useful this blood derived component is for the pathologies referred to here, or for others in which the use of this component may be indicated.

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Table 1

Protein activities in control plasma and protein retention in plasma treated with riboflavin and UV light.

Protein	Reference range	Control (Mean ± SD)	Protein retention in treated plasma (Mean ± SD)
Fibrinogen (mg/dL)	145–385	309 ± 51	67 ± 10%
Factor II (IU/mL)	0.65–1.54	1014 ± 0.10	87 ± 5%
Factor V (IU/mL)	0.54–1.45	1.28 ± 0.18	82 ± 7 = %
Factor VII (IU/mL)	0.62–1.65	1.24 ± 0.18	85 ± 7%
Factor VIII (IU/mL) clotting	0.50–1.50	0.96 ± 0.27	70 ± 6%
Factor VIII (IU/mL) chromogenic	0.45–1.68	0.96 ± 0.24	81 ± 9%
Factor IX (IU/mL)	0.45–1.48	1.02 ± 0.16	82 ± 11%
Factor X (IU/mL)	0.68–1.48	1.09 ± 0.12	85 ± 5%
Factor XI (IU/mL)	0.42–1.44	1.14 ± 0.17	68 ± 8%
Factor XIII (IU/mL)	0.60–1.69	0.86 ± 0.18	113 ± 17%
Antithrombin III (IU/mL)	0.72–1.45	1.02 ± 0.06	98 ± 7%
Protein C (IU/mL)	0.58–1.64	1.07 ± 0.10	98 ± 11
Protein S (IU/mL)	0.56–1.68	1.04 ± 0.13	98 ± 6
α-2 Antiplasmin (IU/mL)	0.72–1.32	1.00 ± 0.08	97 ± 6
vWF antigen (IU/mL)	0.50–1.50	1.10 ± 0.32	99 ± 6
vWF multimers	–	Normal	Abnormal*
vWF protease (ADAMTS-13)	67–177	97 ± 13	88 ± 19
Total protein	48–64	61.5 ± 2.2	101 ± 2

*The abnormal results reported for vWF multimers was a loss of MW multimers.