

# THERAFLEX MB Plasma

## PATHOGEN INACTIVATION SYSTEM FOR PLASMA

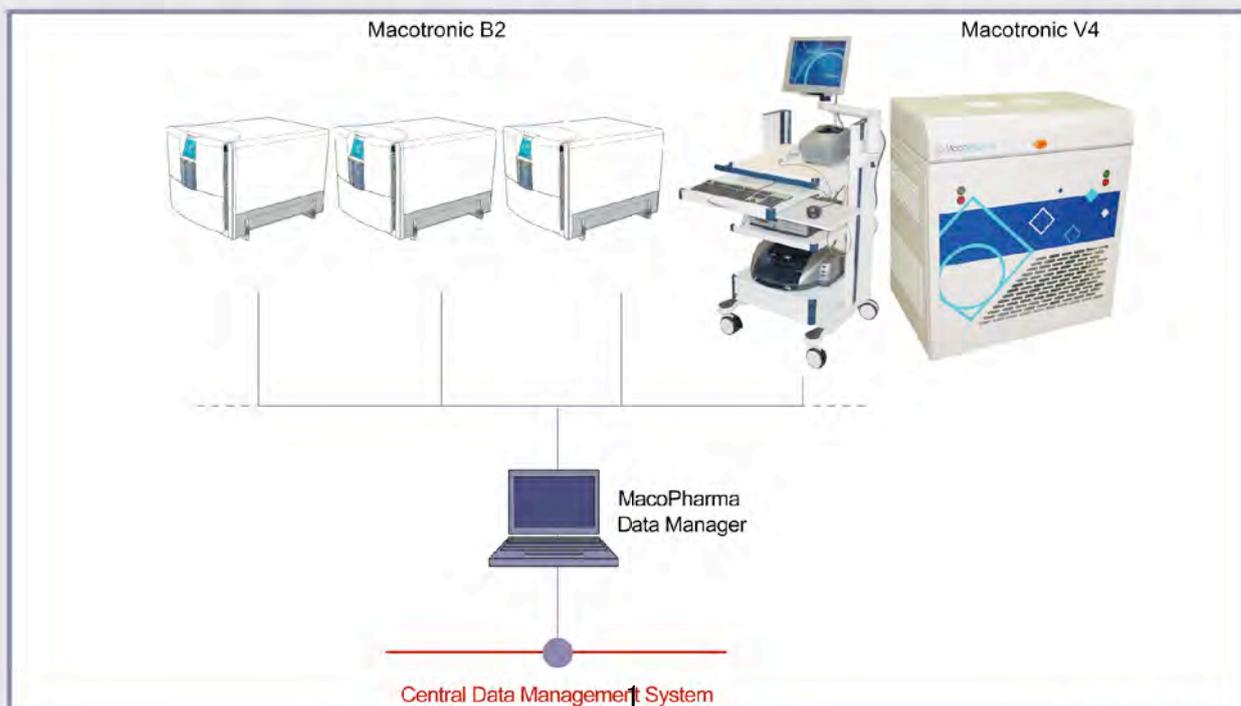
Contributing to 10 years of safer Pathogen Inactivated Plasma

- Routine use worldwide: over 4 million MB Plasma units transfused
- Proven clinical efficacy
- In-house processing
- User friendly technology

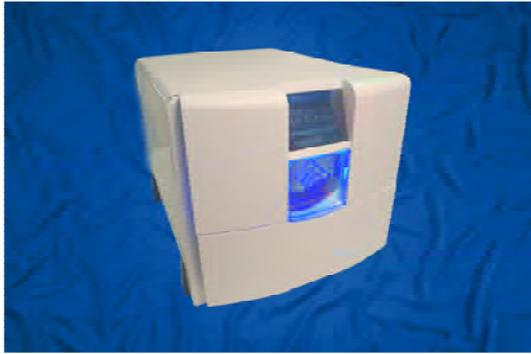
### New Developments:

Macotronic B2, next generation of illumination equipment

- New light source: LED (Light-Emitting Diode)
- Optimal wavelength (630nm)
- Reduced illumination time (15mn)
- Touchscreen operating system
- Integrated RFID technology
- Full GMP-Procedure
- CE Marked by December 2007



# MacoTronic B2



Plasma illumination device for  
the THERAFLEX-MB Plasma  
pathogen reduction process

Ref. 9MB2000

## Specifications :

Dimensions :	47 x 68 x 44 cm (width x depth x height).
Weight :	~ 35 kg
Power supply :	110-240V, 50-60Hz
Lighting system :	96 light-emitting diodes (LED), 4 modules of 24 LED each, 2 modules per bag (double-side exposure)
Wave length :	627 +/-10 nm
Connectivity :	4 USB ports (rear panel : 3, front panel : 1), 1 Ethernet port (network connection)
Screen :	5,5 inch VGA colour touch screen
Cooling system :	Ventilation of illumination chamber by laminar air flow

### • Operating mode :

Delivered energy :	Preset as per THERAFLEX-MB Plasma procedure
Bag loading mode :	Manual opening of the drawer
Capacity per cycle :	2 bags per cycle
Temperature of use :	Air-conditioned room (20-22°C)

### • Operating controls :

Light sensors :	4 control photo-diodes (1 per light module)
Temperature sensors :	2 pyrometers for direct measurement on each bag surface and for ambient temperature in the illumination chamber
Alarms :	Flashing logo for operating status, sound alarm for errors

### • Process control & traceability :

Barcode reading :	Bag ID and batch, product code, operator, post-labelling barcode control
Report printing :	Cycle illumination report providing energy, intensity and temperature records
Cycle record :	Up to 8000 illuminations files stored in the internal memory
Backup :	Transfer of illumination files by USB key or MacoTrace (Data Manager)
Network connection :	TCP/IP protocol, assignable IP address through MacoTrace
Import/export :	Connection to the IT Management System (LIS) through MacoTrace

### • Accessories :

USB barcode laser reader, thermal transfer label printer, report printer, MacoTrace licence, ethernet cable, RFID module

### • Regulations :

CE Marking :	Conformity with MDD 93/42/EEC expected for end Q1 2008
Electrical safety :	Conformity with EN 61010-1 expected for end Q1 2008
Electromagnetic certification :	Conformity with EN 61326-1 expected for end Q1 2008

9MB2000\_A\_C 22/02/2008

# First investigations on a newly developed LED illumination device for the treatment of MB-plasma

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## Purpose

Treatment of fresh frozen plasma (FFP) with MB (methylene blue) and light is a procedure used for the inactivation of blood-borne viruses for more than 15 years. MB plasma is currently produced in several European countries using the MacoPharma Theraflex MB plasma system. High-intensity, long-lived light-emitting diodes (LEDs) are now available on the market, which might replace the sodium vapour lamps currently used in the Macotronic device (MacoPharma). The purpose of this study was the first evaluation of a newly developed LED illumination device (picture 1) with respect to virus inactivation capacity and plasma quality.

Picture1: Prototype



## Methods

Picture 2: Pre-series device



Treatment was done in the Theraflex MB plasma bag system (MacoPharma), containing leucocyte-depletion filter Plasmaflex, MB pill (85 µg MB), illumination bag, MB depletion filter Blueflex and plasma storage bag. For investigating plasma quality 3 plasma pools were each prepared from 3 different single donor units. Plasma was divided into three illumination bags and illuminated on the LED device. Samples were taken at different time points of illumination and factor VIII and fibrinogen (Clauss) were determined.

For investigating virus inactivation capacity Pseudorabies virus (PRV) was spiked into FFP (n = 4) resulting in a titer of approximately 10<sup>6</sup> tissue culture infectious doses/ml (TCID<sub>50</sub>/ml). The viral titer was determined from samples taken after 5, 10, 15 and 20 minutes of illumination on the LED device. Infectivity was determined by endpoint titration using a Vero cell CPE (cytopathic effect) assay. In a preliminary run the currently used Macotronic was compared with the newly developed LED device.

## Results

Virus inactivation was investigated using the PRV, an enveloped, double-stranded DNA virus, which is used as model virus for HBV. MB/light treatment using the LED-based illumination device resulted in an inactivation of > 4 log steps of PRV after 10 – 15 min of illumination (diagram 1). The device is at least as effective as the Macotronic device routinely used at present.

Table 1: Inactivation kinetics of PRV (n = 4)

sampling time [min]	0	5	10	15	20
log <sub>10</sub> TCID <sub>50</sub> /ml (mean ± SD)	6.16 ± 0.23	4.64 ± 0.68	≤ 2.41 ± 1.65	≤ 1.75 ± 0.38	≤ 1.54 ± 0.00
log <sub>10</sub> reduction factor		1.52	≥ 3.75	≥ 4.41	≥ 4.62

Plasma quality was only slightly affected by illumination. Factor VIII was decreased by 17% and fibrinogen (Clauss) by 14% during an illumination of 20 min (Diagram 2). Results are at least comparable to or might even be better than those for the Macotronic device.

Diagram 1: Inactivation of PRV by LED device (n=4) and Macotronic (n=1)

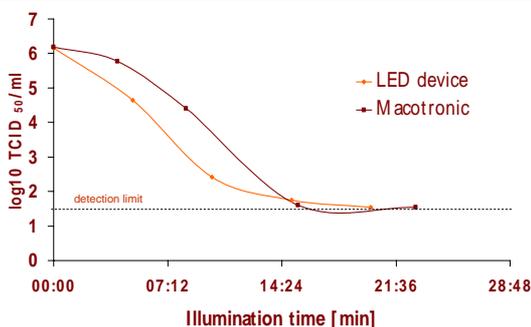
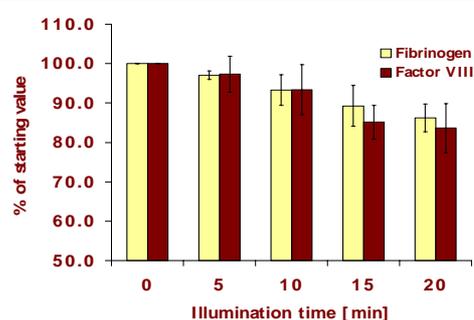


Diagram 2: Influence on coagulation factors (n = 3)



## Conclusions

A new, compact illumination device based on long-lived LEDs was developed. The preliminary data suggest that this LED device is comparable to the Macotronic device with respect to virus inactivation capacity and preservation of plasma quality. Illumination time might even be shortened by using this high intensity illumination device.

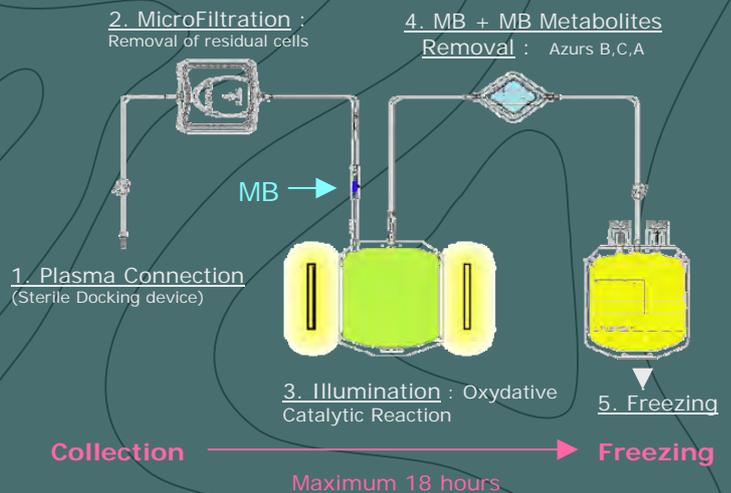
# Three years' haemovigilance of methylene blue-treated fresh frozen plasma : no increase in transfusion reaction incidence

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<sup>2</sup> Service Francophone du Sang, Belgian Red Cross, BELGIUM

## Background

Due to stringent donor selection, laboratory testing and pathogen inactivation procedures, fresh frozen plasma (FFP) offers a high degree of viral safety. In Belgium, only non remunerated volunteers are recruited as plasma, platelets and blood donors. Pathogen inactivation is a proactive strategy designed to inactivate a pathogen before it enters the blood supply. Methylene blue-photoinactivation was chosen by the Belgian Red Cross two years ago (Theraflex MB plasma – MacoPharma)

## MB-viroinactivation (Theraflex-MB plasma MacoPharma)



## Methods

Until mid 2004, only solvent-detergent FFP (SD-FFP) was used in our teaching hospital. BM-FFP began to be transfused to our patients thereafter. Since nearly 10 years, all transfusions of labile blood products are checked for the appearance or not of an adverse event. In this study, the incidence and the seriousness of adverse event after BM-FFP transfusion were compared with those observed with SD-FFP.

## Results

- (a) : benign allergic reaction (n=4)  
 moderate allergic reaction (n=1)  
 NHFR (n=1)
- (b) : benign allergic reaction (n=5)  
 moderate allergic reaction (n=3)
- No TRALI episode (any previous pregnancy / transfusion = contra-indication for plasma donation)

	SD-FFP	BM-FFP
Period	2003 – 2004	mid 2004 – 2005
Number of FFP units transfused	5101	5660
Number of adverse reactions after FFP Tf°	6 (a)	8 (b)
Incidence of reaction	0.12 %	0.14 %

## Discussion and conclusion

No significant increase in adverse event incidence was observed after BM-FFP transfusion (odds ratio 1.2) and the seriousness of these events was comparable. The clinical efficacy of both FFP was similar : both procedures have limited effects on coagulation factors (especially on fibrinogen and factor VIII for MB, on protein S and alpha 2-antiplasmin for SD). Finally, plasma pooling may have the undesirable effect of increasing the risk of transmitting viruses that are either resistant to the process or have escaped the virucidal process. The hallmark of MB technology is that it allows the viral inactivation of single donor units of FFP, offering reassurance that no increased infectious risks are added due to pooling. This a crucial point in term of public health safety.

# Safety Of Methylene Blue Treated Plasma

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## OBJECTIVES

The procedure using methylene blue (MB) to inactivate viruses in therapeutic plasma is well established worldwide. It includes membrane filtration (Plasmaflex, 0.65 µm pore size), addition of MB (dry pill, 85 µg, resulting in 1 µmole/L at 266 ml), illumination (approx. 20 min, 590 nm), and filtration of MB and photoproducts (Blueflex). More than 4 million units of plasma were transfused without any unusual adverse event reported.

Aim of this study was to prove the toxicological safety of MB, its photoproducts azure A, B and C, and that of MB-treated plasma.

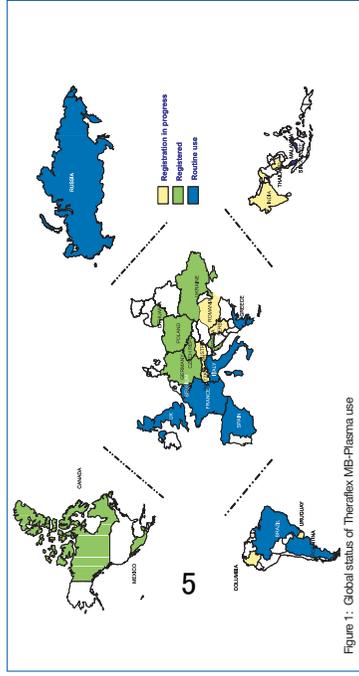


Figure 1: Global status of Theralex MB-Plasma use

## METHODS

Adsorption, distribution and excretion of <sup>14</sup>C-labeled MB following 24 h infusion were investigated at a dose level of 20 mg/kg body weight (b.w.) in rats. Observation time was 96 hours.

Studies on teratogenic effects were done by intravenous bolus injection of MB into rats and Beagle dogs. MB was administered daily to the dams at 4, 12, 36 mg/kg b.w. (rat) and 2, 6, 18 mg/kg b.w. (rabbit).

In a tolerance test 5 ml/kg b.w. of autologous light-treated plasma (1 or 10 µM MB) was administered to 5 male Beagles per group by intravenous administration. After 21 d 3 dogs/group were treated again and sacrificed 24 h later. Hematology, clinical biochemistry, and electrocardiogram were examined. A complete histopathology was done.

MB and Azure A/B/C were tested in: bacterial reverse mutation test (Ames test), in vitro mammalian cell gene mutation test, in vitro mammalian chromosome aberration test with human lymphocytes, in vivo micronucleus test with rat bone marrow and peripheral blood cells (20 mg/kg b.w., 24 h infusion), in vivo unscheduled DNA synthesis test in rats (20 mg/kg b.w., bolus infusion).

## RESULTS

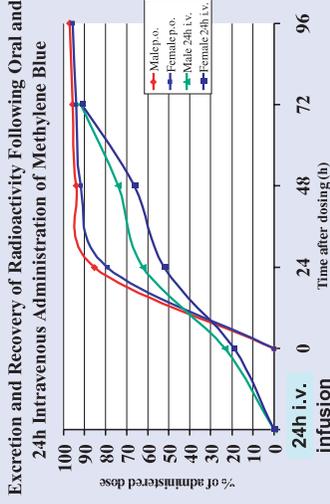


Fig. 2 Recovery of <sup>14</sup>C-labeled methylene blue (MB). The recovery was examined in Sprague-Dawley rats following oral administration (p.o.) and 24 h i.v. infusion at a dose level of 20 mg MB/kg body weight. Urine, faeces, organs, expired air, rinse water and infusion site were analysed. The radioactivity recovery rate in organs and at the infusion.

- Pharmacokinetics of <sup>14</sup>C-labeled MB after 24 h infusion were determined in T<sub>max</sub>, T<sub>1/2</sub> α and T<sub>1/2</sub> β It indicated:
  - biphasic elimination of MB with an initial half-life of 3 min and a longer terminal half-life of 12.6 h (male) and 16.0 h (female)
  - less than 1 % radioactivity in plasma and examined organs
  - Excretion of radioactivity was almost complete after 96 h
  - no accumulation or storage of MB
- The no observed effect level (NOEL) for the fetal organism was 4 mg and 6 mg/kg b.w./day in rats and rabbits.
- Clastogenic effects of MB and Azure B were found in vitro.
- No genotoxic effects on bone marrow, peripheral blood cells and hepatocytes after application of 20 mg/kg b.w. MB and Azure B.
- No signs of intolerance or sensitization after infusion of 1 µM or 10 µM MB light-treated plasma before removal of MB and photoproducts were observed.

Distribution of Radioactivity 96h After Administration of <sup>14</sup>C-Methylene Blue

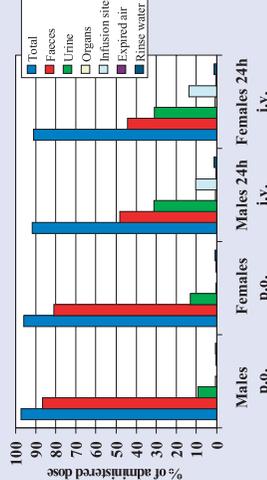


Fig. 3 Mean recovery of radioactivity after oral (gavage) application and 24 h i.v. infusion of 20 mg MB/kg body weight in rats.

Safety Margins for Toxicity from *in vivo* Studies with Methylene blue and Methylene Blue Treated Plasma

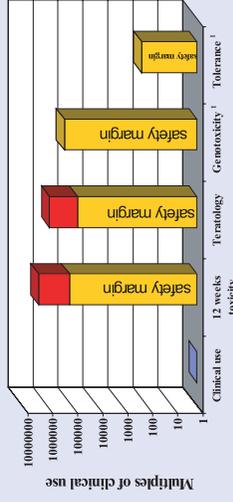


Fig. 4 Safety margins for toxicity from *in vivo* studies with methylene blue. Calculations are based on the no-observed effect level (slight methaemoglobinemia) (12 weeks toxicity) and a normal clinical exposure of 0.1 µg MB/kg body weight (1-6 units MB plasma). No toxicity occurred; therefore the safety margins are based on the highest dose tested.

## CONCLUSIONS

Thresholds for no or low toxic properties which occurred after administration of MB in preclinical studies varied depending on the amount of MB applicable in the specific test system. They are > 160 to 200,000 fold higher than the estimated clinical exposure of MB after infusion of 6 units MB-light treated plasma.

## REFERENCES

- Williamson et al. 2003 Transfusion 43:1322-1329
- Pohler et al. 2004 Transfus Med Hemother 31 (suppl 3):1-84, PS05

# CHARACTERISTICS OF MACO PHARMA THERAFLEX MB-PLASMA

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ISBT Congress, Madrid, June 2007

**Background:** During the last 15 years the method using methylene blue (MB) to inactivate viruses in plasma was constantly improved. Invented by the Blood Center of the German Red Cross, chapters of NSTOB, Institute Springe, the initial procedure included: Freezing and thawing to release intracellular viruses from leucocytes, addition of a proportional amount of a MB stem solution to a final concentration of 1 µM, and subsequent one-side illumination for one hour with fluorescent tubes.

**Aim:** The aim was to improve the original method to facilitate the implementation in the blood bank.

## Results

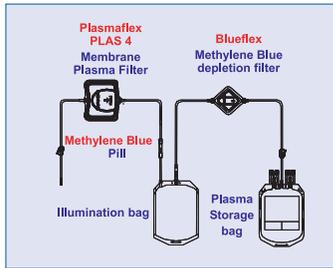


Figure 1: bag system



Figure 2: illumination device Macotronic

### Plasma quality

Test	Unit	Range	Init. value	after prep.	3 month	9 month	18 month	27 month
<b>Global test</b>								
Thrombin time	[s]	14 - 21	15,2 ± 0,3	17,4 ± 0,9	15,9 ± 1,1	16,2 ± 1,1	18,5 ± 0,5	20,2 ± 0,9
<b>Coagulation factors</b>								
Fibrinogen (Clauss)	[mg/dl]	200 - 450	262,3 ± 8,5	190,5 ± 12,6	194,0 ± 14,0	190,8 ± 11,3	254,5 ± 13,4	227,0 ± 11,7
Factor II	[%]	70 - 130	104,8 ± 2,1	101,9 ± 2,6	96,6 ± 1,4	103,0 ± 5,3	109,3 ± 5,2	104,5 ± 2,9
Factor V	[%]	60 - 130	87,1 ± 6,0	105,6 ± 8,8	108,1 ± 3,8	105,0 ± 8,8	99,4 ± 6,4	107,6 ± 3,8
Factor VIII	[%]	60 - 150	88,6 ± 17,9	72,5 ± 15,7	82,3 ± 17,6	72,9 ± 13,8	73,5 ± 14,8	81,5 ± 13,1
Factor IX	[%]	60 - 130	100,4 ± 5,8	92,8 ± 3,1	90,9 ± 6,8	97,8 ± 4,8	77,5 ± 5,3	99,6 ± 8,1
Factor XI	[%]	60 - 130	98,6 ± 5,4	78,4 ± 7,1	80,1 ± 4,5	79,3 ± 5,9	71,5 ± 4,3	87,5 ± 3,4
vWF:RCo	[%]	60 - 150	96,5 ± 5,3	100,5 ± 15,5	110,3 ± 20,7	112,8 ± 22,2	101,8 ± 15,8	110,8 ± 19,8
<b>Inhibitors</b>								
free Protein S	[%]	55 - 130	104,3 ± 6,4	103,5 ± 7,0	81,8 ± 7,2	98,8 ± 10,5	99,0 ± 5,9	98,8 ± 7,0
Protein C	[%]	70 - 140	97,8 ± 7,7	89,5 ± 5,9	85,0 ± 7,6	81,0 ± 18,5	114,0 ± 10,3	97,0 ± 6,5
AT III	[%]	80 - 120	91,3 ± 3,3	90,5 ± 3,3	95,5 ± 2,6	91,8 ± 3,8	110,8 ± 4,1	102,0 ± 6,8
<b>Fibrinolysis</b>								
α <sub>1</sub> -Antitrypsin	[mg/dl]	90 - 200	98,5 ± 1,3	97,5 ± 2,5	98,3 ± 1,5	99,3 ± 2,1	106,5 ± 2,4	100,8 ± 3,4
α <sub>2</sub> -Antiplasmin	[%]	80 - 120	95,0 ± 2,8	94,0 ± 2,6	92,8 ± 4,4	84,8 ± 2,1	96,0 ± 4,2	100,5 ± 4,4
<b>Activation</b>								
Factor XIIIa	[mU/ml]	< 50	31,3 ± 4,1	33,3 ± 3,9	33,4 ± 6,2	35,1 ± 6,4	36,3 ± 6,8	36,3 ± 4,7
<b>Complement</b>								
CH100	[U/ml]	392 - 1019	689,6 ± 157,4	579,1 ± 31,7	949,3 ± 85,7	771,3 ± 161,5	798,8 ± 179,7	978,0 ± 74,1

Figure 3: Plasma quality after treatment during storage for 27 month

### Virus reduction capacity

Sensitivity of enveloped viruses		
Virus	Family	Reduction rate (log <sub>10</sub> )
HIV-1	Retro	5,45 <sup>2</sup>
WNV	Flavi	5,78 <sup>+2</sup>
BVDV	Flavi	5,44 <sup>+2</sup>
Hog cholera	Flavi	5,92 <sup>+1</sup>
PRV	Herpes	5,48 <sup>+2</sup>
Herpes Simplex	Herpes	5,50 <sup>+1</sup>
Bovine herpes	Herpes	8,11 <sup>+1</sup>
Semliki Forest	Toga	7,00 <sup>+1</sup>
Sindbis	Toga	9,73 <sup>1</sup>
Influenza	Orthomyxo	5,1 <sup>1</sup>
HBV (Duck model)	Hepadna	> 6 <sup>3</sup>
Vesicular Stomatitis	Rhabdo	4,89 <sup>+1</sup>
Sensitivity of non-enveloped viruses		
Virus	Family	Reduction rate (log <sub>10</sub> )
Adeno	Adeno	4 <sup>1</sup>
Calici	Calici	3,9 <sup>+1</sup>
SV 40	Papova	4 <sup>1</sup>
Parvo B 19	Parvo	5 <sup>1</sup>

<sup>1</sup> Mohr et al. Immunological Investigations 1995, 24(1&2); 73-85  
<sup>2</sup> tested by Analysis Biomedizinische Test GmbH  
<sup>3</sup> tested by Prof. Christian TREPO et al., INSERM Unit 271, Lyon, France  
<sup>4</sup> tested under production conditions  
Reduction below the limit of detection

Figure 4: Virus reduction capacity

### Safety margins for toxicity from *in vivo* studies with MB and MB-treated plasma

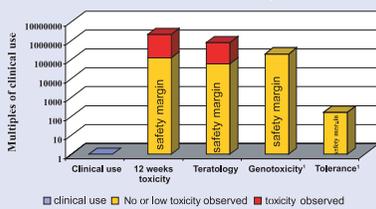


Figure 5: Safety margin for toxicity of Methylene Blue

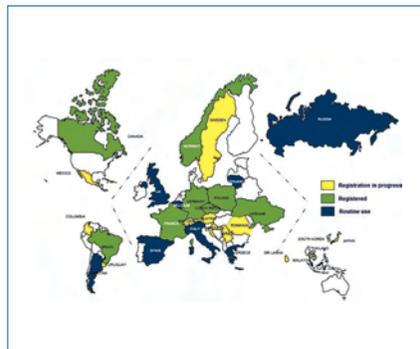


Figure 6: Global status of Theraflex MB-Plasma use

### Methylene blue reduction by Blueflex filtration

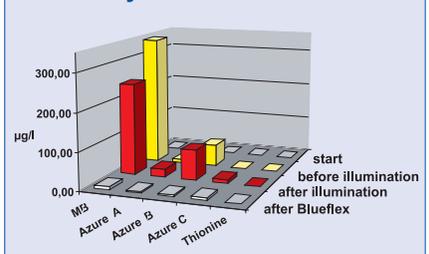


Figure 7: Methylene Blue reduction by Blueflex filtration

**Results:** With the current Theraflex MB-Plasma procedure provided by MacoPharma the procedure is markedly improved. The elimination of leucocytes is realized by membrane filtration, MB is added as an integrated dry pill, and residual MB and photoproducts are removed by a special Blueflex filter. The specially designed illumination device (Macotronic) ensures treatment under GMP conditions. Illumination dose and intensity are constantly monitored and temperature is controlled. The use of sodium low pressure lamps as improved light sources allowed the reduction of the illumination time to about 20 min.

The characteristic features of the system are:

1. Virus inactivation of enveloped viruses shows a reduction rate of at least 5 log<sub>10</sub> steps. (Figure 4)
2. Plasma quality: Only fibrinogen and factor VIII are reduced by about 20-25%. (Figure 3)
3. Clinical use: More than 4 million MB-treated plasmas were transfused with excellent tolerance and efficacy in several countries all over the world. (Figure 6)
4. MB and photoproducts are eliminated by more than 90% using the Blueflex filter. (Figure 7)
5. Toxicology: Investigation on toxicology of MB and photoproducts showed a high safety margin for the concentration used. (Figure 5)

## Conclusions

**Conclusions:** The MacoPharma Theraflex MB-Plasma represents an efficient, safe, and easy to use system which generates virus-safe plasma of high quality.

# THE EFFECT OF METHYLENE BLUE PATHOGEN REDUCTION SYSTEM ON Fc VIIIc IN PLASMA DERIVED FROM WHOLE BLOOD DURING STORAGE

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<sup>1</sup> Dienst voor het Bloed, Rode Kruis-Vlaanderen, Belgium.

<sup>2</sup> Service du Sang, Croix Rouge de Belgique, Belgium.

## BACKGROUND

The virucidal properties of methylene blue have been documented since 1930. In 1991, the Springe Institute developed a photodynamic method to inactivate pathogens, particularly viruses, in human plasma using methylene blue in combination with visible light. MacoPharma has improved this method and developed the THERAFLEX MB-Plasma system consisting of the Macotronic illumination machine, together with an appropriate disposable set for pathogen reduction and removal of residual methylene blue to a level less than 4µg/unit.

This method is known to be effective on viruses as well as other documented pathogens, although reducing slightly the activity of clotting factors such as factor VIII (Fc VIIIc) and Fibrinogen. According to Belgian legislation, plasma should be frozen within 18 hours after blood collection. In the case of pathogen reduced plasma, a level of at least 0.5 IU / ml for Fc VIIIc should be attained.

The aim of this study was to investigate the effect on Fc VIIIc recovery of various time delays, between donation and the photodynamic treatment of plasma derived from whole blood.

## MATERIALS AND METHODS



In centre 1, 143 units of whole blood were selected from volunteer male A+ donors. These units were divided into 3 groups. Plasma from group A was separated at 4 hours and treated at 5.5 hours, group B plasma at 4 and 16.5 hours, and group C plasma at 15 and 16.5 hours, respectively.



In centre 2, 120 units of whole blood were selected from volunteer male or female donors of any blood group. These units were also divided into 3 groups. Plasma from group D was separated at 3.5 hours and treated at 8 hours, group E plasma at 3.5 and 11 hours, and group F plasma at 12.5 and 16.5, respectively.



Pathogen reduction was performed using the THERAFLEX MB-Plasma system (disposable with

leucodepletion filter and methylene blue removal filter, Macotronic illumination machine). Samples for Fc VIIIc activity assay were taken immediately after separation and after treatment. Fc VIIIc measurement was done using a one-stage aPTT clotting assay with Fc VIIIc deficient plasma. Results of Fc VIIIc recovery are expressed in percentage of activity.



Prior to separation and photo treatment, the whole blood and plasma were stored on eutectic plates to keep the products at a temperature of 20 °C. Results were analysed using repeated measures Anova for general comparison, student t-test for group comparison and paired student t-test when appropriate.



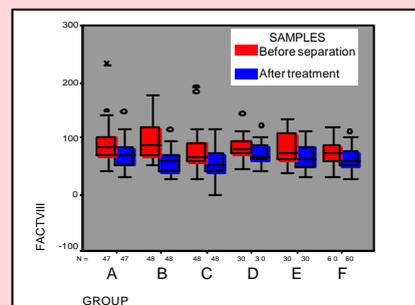
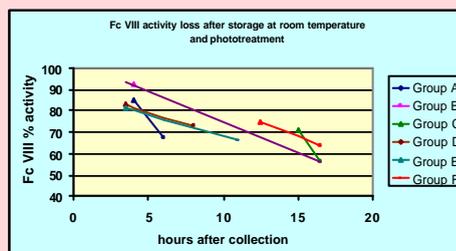
## RESULTS

Fc VIIIc pre- and post-photo treatment mean results are presented in table 1.

	Time before separation (h)	Time before treatment (h)	Pre treatment <sup>1</sup> Fc VIIIc (%)	Post treatment <sup>2</sup> Fc VIIIc (%)
Group A	4	5.5	85	67
Group B	4	16.5	92	56
Group C	15	16.5	71	56
Group D	3.5	8	83	73
Group E	3.5	11	81	66
Group F	12.5	16.5	75	64

Table 1 : <sup>1</sup> Sample taken after separation <sup>2</sup> Sample taken after treatment

- The difference between the two groups was statistically significant ( $p < 0.001$ ).
- A significant decline in Fc VIIIc activity was measured in all groups following the photo treatment process ( $p < 0.001$ ).
- No significant difference between group B and groups C and F after photo treatment was observed ( $p = 0.87$ ,  $p = 0.10$ ); this suggests no significant difference in the loss of Fc VIIIc activity between the time of separation of whole blood into plasma and photo treatment 16.5 h.
- No significant difference between Fc VIIIc activity loss in group A and C ( $p = 0.66$ ) and in group D and F ( $p = 0.53$ ) suggesting that the time interval between blood collection and separation does not influence the loss of Fc VIIIc activity post photo treatment.



## CONCLUSION

Regarding the Fc VIIIc activity of plasma, Methylene blue pathogen reduction has to be completed within a limited time interval after whole blood donation. The processing / separation of whole blood can be performed at any time between donation and the photo treatment of plasma. Following the attainment of these results, methylene blue pathogen reduction of plasma has been implemented in both centres.

# Quality Control Evaluation of Methylene Blue Light Treated Plasma.

L Larrea<sup>1</sup>, A Cerveró<sup>2</sup>, M Calabuig<sup>1</sup>, A Blanquer<sup>1</sup>, P Solves<sup>1</sup>, V Mirabet<sup>1</sup>, R J Roig<sup>1</sup>.  
<sup>1</sup>Centro De Transfusión De La Comunidad Valenciana, Valencia, Spain;  
<sup>2</sup>H General Universitario, Valencia, Spain.

## INTRODUCTION

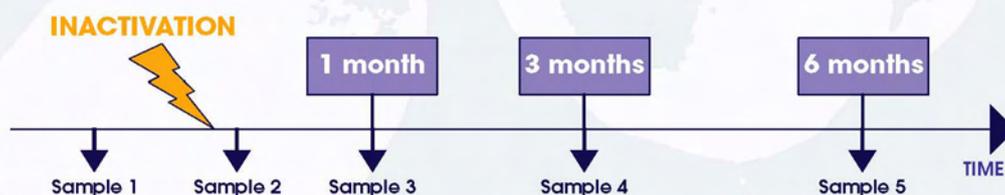
Methylene blue light treated plasma has been used in Spain from 1998. Since then slight variations of the technique have been implemented such as plasma leukoreduction instead of freezing and thawing. Studies on plasma quality have been published analysing initial methods. We have tested several plasma units to evaluate plasma quality with the current technology.

## MATERIAL AND METHODS

Briefly speaking, 450±50 mL whole blood units were collected in automatic scales using top & bottom blood bags. After collecting, units were cooled with 1.4- butanodiol plates and later stored at 22±2°C. Then, whole blood was centrifuged at high speed to obtain a concentrate of red blood cells and plasma, while maintaining the buffy coat in the initial blood bag. For plasma inactivation the Springe modified method was used (Theraflex-MB-Plasma system: Macopharma®). Plasma was joined to the MB system by means of sterile docking and, simultaneously, gravity filtered. In batches of four plasma was illuminated for 20 min. Units for storage were frozen after inactivating before 24 hour postdonation. For the study we inactivated 30 plasma units (10 A, 10 O, 5 B and 5 AB). We took several samples: before inactivation (sample1), just after inactivation (sample2), after 1 month of storage at -30°C (sample3), after 3 months of storage at -30°C (sample4) and after 6 months of storage at -30°C (sample5). After each moment samples were stored at -80°C till the tests were performed. In every sample we performed the following tests: PT, APTT, FV, FVIII and fibrinogen.



## The process of the 5 plasma samples



## RESULTS

As published before most affected parameters by the inactivation procedure were fibrinogen and FVIII (18 and 16% respectively decrease from sample 1 to 2). FV was scarcely affected (a 3% decrease from sample 1 to 2). PT an APTT were prolonged only in 2.74 and 5.26% respectively from sample 1 to 2. Results may be seen with more detail in the attached table.

## Results of the tests (PT, APTT, Fib, FV and FVIII) for the 5 plasma sample

Sample parameter	(%) ↑ PT	(%) ↑ APTT	(%) ↓ Fib	(%) ↓ FV	(%) ↓ FVIII
△ 1-2	2,74	5,26	18,67	3,4	16,9
△ 1-3	6,16	3,85	23,31	5,95	21,04
△ 1-4	7,99	6,23	23,79	13,64	25,62
△ 1-5	13,54	4,6	25,63	14,88	26,39

PT: Prothrombin rate  
 FV: Factor V

APTT: Activated Partial Thromboplastin Time test  
 FVIII: Factor VIII

Fib: Fibrinogen

## CONCLUSIONS

The Methylene blue inactivation methodology is very easy to use and the plasma factors after inactivation are preserved. However, during storage there is a certain loss of coagulation factors. If the reason for this is related to the treatment or the storage conditions remains to be evaluated.

# THERAFLEX MB-PLASMA PROCEDURE: PLASMA QUALITY AFTER 15 MONTH STORAGE

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ISBT Congress, Cape Town, September 2006

## Introduction

**Background:** Although in the last decades thanks to the implementation of several methods like donor selection and testing procedures the risk of virus transmission from plasma has decreased, infection of patients still exists. Additionally new viruses like West Nile Virus enter the transfusion chain. Therefore, the treatment of therapeutic plasma with methylene blue (MB) is a technique used in several European countries for virus inactivation. MacoPharma has developed the proprietary photodynamic Theraflex MB-Plasma bag system including a MB pill, an illumination system (Macotronic) with visible light, and a final MB filtration step with the Blueflex filter (Williamson et al. Transfusion 2003;43:1322-1329).

**Aims:** Aim of this study was to show the reduction of MB and photoproducts due to the Blueflex filter and to prove the reproducibility of the filtration efficiency. Additionally the quality of the plasma after 15 month storage was checked.

## Materials & Methods

18 plasmas were treated at three different days. At different steps of the Theraflex MB-Plasma procedure the MB and photoproduct content was measured by HPLC, which was described previously (Verpoort et al. 2003; ISBT Istanbul P246). Measurement was done after dissolution of the MB pill, after illumination, and after filtration (Fig. 2).

At each day plasma was pooled and divided into several storage bags (storage temperature <-30 °C). At different time points a palette of plasma factors was measured.

1. global tests (Quick, INR, aPTT, thrombin time)
2. coagulation factors (Fibrinogen, F II, F V, F VII, F VIII:c, F IX, F X, F XI, F XII, FXIII, vWF Ristocetin Co-Factor)
3. Inhibitors (AT III, Protein C, Protein S)
4. Fibrinolysis (Plasmin inhibitor, alpha-1-Antitrypsin, Plasminogen)
5. Complement (CH50, C3a)
6. Activation (TAT, F XIIa, D-Dimer)

## Results

Test	Limits	Unit	0 month	6 month	9 month	15 month
<b>1. Global tests</b>						
Quick	80 - 130	%	98 ± 2	105 ± 3	94 ± 2	93 ± 3
INR	1.0 - 1.30		1.0 ± 0.0	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
aPTT	30 - 60	sec	34 ± 1	35 ± 1	34 ± 1	35 ± 1
Thrombin time	14 - 21	sec	22.7 ± 0.7	23 ± 1.0	23 ± 1.0	22 ± 1.0
<b>2. Coagulation factors</b>						
Fibrinogen	1.8 - 3.5	g / l	2.4 ± 0.1	2.3 ± 0.2	2.4 ± 0.2	2.4 ± 0.1
F II	70 - 130	%	99 ± 4	104 ± 4	106 ± 7	105 ± 12
F V	65 - 150	%	101 ± 3	99 ± 6	103 ± 9	101 ± 10
F VII	70 - 130	%	103 ± 8	119 ± 9	108 ± 3	102 ± 4
F VIII:c	0.50 - 2.00	I.U. / ml	0.81 ± 0.15	0.83 ± 0.12	0.92 ± 0.16	0.87 ± 0.11
F IX	0.70 - 1.30	I.U. / ml	1.00 ± 0.05	0.92 ± 0.04	0.98 ± 0.04	0.94 ± 0.04
F X	70 - 130	%	106 ± 6	108 ± 5	111 ± 7	98 ± 3
F XI	50 - 130	%	82 ± 2	92 ± 3	83 ± 3	83 ± 4
F XII	70 - 130	%	87 ± 10	101 ± 11	99 ± 10	88 ± 11
F XIII	70 - 130	%	81 ± 11	77 ± 7	75 ± 4	85 ± 9
vWF (Ristoc. Co-F)	50 - 140	%	85 ± 8	81 ± 19	94 ± 13	95 ± 7
<b>3. Inhibitors</b>						
AT III	0.80 - 1.30	I.U. / ml	1.12 ± 0.08	1.02 ± 0.08	1.06 ± 0.07	0.99 ± 0.06
Protein C	70 - 130	%	110 ± 7	109 ± 6	116 ± 6	110 ± 3
Protein S	70 - 140	%	75 ± 7	70 ± 2	76 ± 2	81 ± 2
<b>4. Fibrinolysis</b>						
Plasmin inhibitor	80 - 120	%	103 ± 4	101 ± 3	103 ± 6	105 ± 10
alpha-1-Antitrypsin	0.70 - 1.50	I.U. / ml	1.14 ± 0.03	1.12 ± 0.07	1.24 ± 0.09	1.20 ± 0.08
Plasminogen	75 - 140	%	102 ± 7	105 ± 8	12 ± 11	104 ± 10
<b>5. Complement</b>						
CH50	70 - 100	%	116 ± 14	110 ± 11	108 ± 5	114 ± 8
C3a	123-2228	ng / ml	1029 ± 323	1106 ± 596	898 ± 332	921 ± 217
<b>6. Activation</b>						
TAT	1 - 4.1	µg / l	2.1 ± 0.2	2.4 ± 0.5	2.0 ± 0.0	2.2 ± 0.7
F XIIa	< 3.0	ng / ml	1.0 ± 0.2	0.9 ± 0.1	1.3 ± 0.6	1.0 ± 0.0
D-Dimer	64 - 246	µg / l	241 ± 120	239 ± 131	199 ± 97	225 ± 122

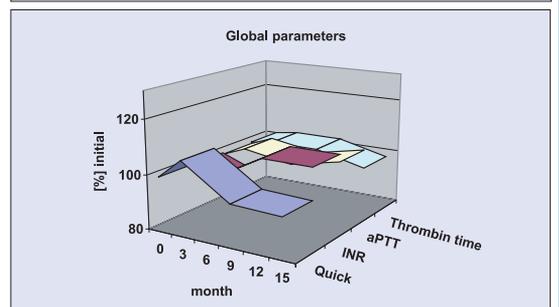
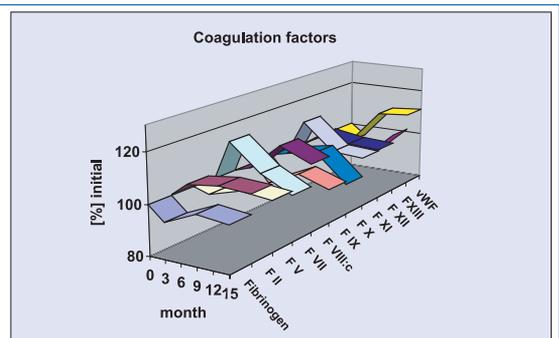


Fig. 1: Storage stability of Theraflex MB-Plasma

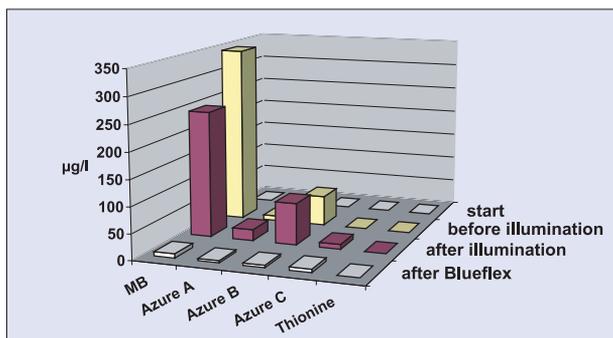


Fig. 2: Methylene blue and photoproduct reduction due to Blueflex filtration

Illumination of MB-containing plasma with visible light using the Macotronic illumination device resulted in the generation of photoproducts as described previously. Mean reduction of the total phenothiazine content was 94.5%. Every single filtration yielded in a filtration efficiency of minimum 91%. The mean reduction capacity for MB was above 99.9%.

There was no significant change in the plasma factor content after treatment during the whole 15 month storage period. Slight variations are within the error of measurement. The only difference between the plasma parameters resulted from the treatment itself. Here, an increase in the INR and aPTT (14.3 %; 18.2 %), decrease of fibrinogen (-19.3 %), factor V (-25.3 %), factor VIII (-21.8 %), factor IX (-25.6 %), factor X (-23.4 %), and factor XI (-16.8 %) was observed. Despite this variations the values were within the ranges found in non-treated plasma.

## Conclusions

The filtration of plasma with the Blueflex filter is a reliable method to reduce the amount of MB and photoproducts substantially. The plasma quality is not changed during the observed storage period and remains within the physiological variation of non-treated plasma.

# QUALITY OF THERAFLEX® MB-PLASMA DURING STORAGE AND TREATMENT

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<sup>2</sup>MacoPharma International GmbH, Langen  
ISBT Congress, Athens, July 2005

## Introduction

**Background:** Although in the last decades thanks to the implementation of several methods like donor selection and testing procedures the risk of virus transmission from plasma has decreased, infection of patients still exists. Additionally new viruses like West Nile Virus enter the transfusion chain [1]. Therefore, the photodynamic treatment of therapeutic plasma with methylene blue (MB) is a technique used in several European countries for pathogen inactivation [2]. MacoPharma has developed the proprietary Theraflex® MB-Plasma bag system including a MB pill and a final MB filtration step.

**Aim:** Aim of the study is to show the quality of the MB plasma during the preparation procedure and during storage using the Theraflex® system (see Figure 1).

## Materials & Methods

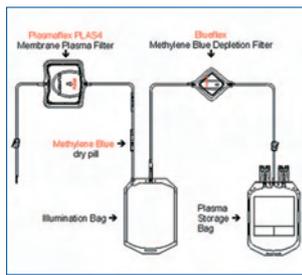


Figure 1: Theraflex® MB-Plasma bag system

### Preparation Process

For the preparation process every single step was evaluated using 18 single donor plasma units. For the evaluation of the plasma factors 5 ml were drawn at different stages (see Figure 2). The samples were pooled after drawing and measured for the specified factors. Six samples of each stage were pooled at three days. A whole panel of plasma factors was measured for the resulting three pools (see Figure 3).

### Stability

Stability data were generated using three plasma pools. Six plasmas were pooled and afterwards divided into six aliquots. Each was treated as single unit and then each was divided into six storage samples. The same plasma factors as for the manufacturing process were evaluated.

MB and photoproduct content was below the detection limit as previously described [3].

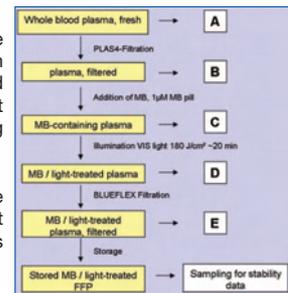
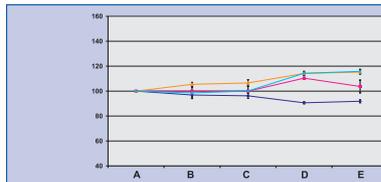


Figure 2: Sampling scheme

## Results

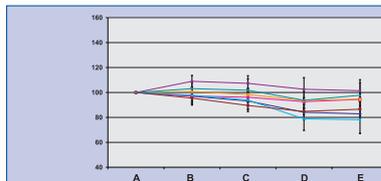
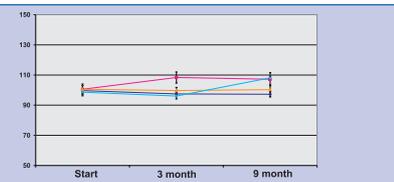
### In-process control



### Global tests

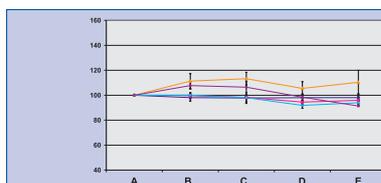
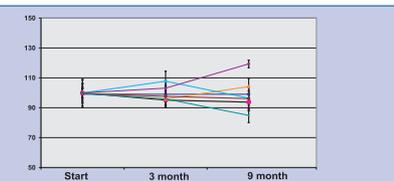
- Quick
- INR
- aPTT
- thrombin time

### Storage stability



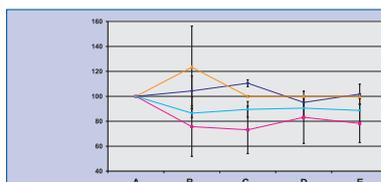
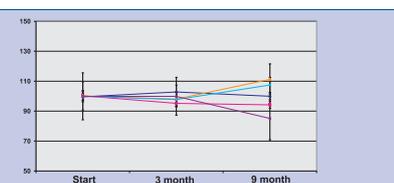
### Coagulation factors

- Fibrinogen
- F II
- F V
- F VIII:c
- F IX
- F X
- F XI



### Coagulation inhibitors

- AT III
- Protein C
- Protein S
- Plasmininhibitor
- alpha-1-Antitrypsin



### Complement activation

- CH50
- TAT
- F XIIa
- D-Dimer

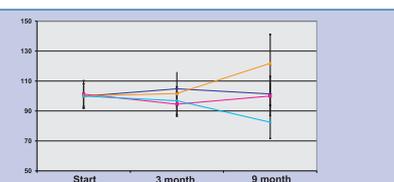


Figure 3: Percentage of deviation from the source plasma for different plasma factors during storage and treatment A: before treatment; B: after PLAS4 filtration; D: after MB addition; E: after Blueflex filtration

All investigated plasma factors remained stable during the investigated storage time. A moderate reduction for some coagulation factors during the preparation was found in the illumination step but not in the other preparation stages. This was mainly fibrinogen (17,5 %), factor VIII (22,2 %), and factor X (13,4 %). Despite this reduction the values were within the ranges found in non-treated plasma.

## Conclusions

Plasma treated with the Theraflex procedure showed slight reduction during treatment and no reduction during storage. All plasma factors remained within the threshold values. The treatment of therapeutic plasma with MB is a valid technique of pathogen inactivation.

[1] West Nile virus in plasma is highly sensitive to methylene blue-light treatment Mohr et al, *Transfusion* 2004;44:886-890

[2] Methylene blue-treated fresh-frozen plasma: what is its contribution to blood safety? LM Williamson et al, *Transfusion* 2003;43:1322-1329

[3] Filtration of Methylene Blue and Photoproducts after Photodynamic Treatment of Plasma using BLUEFLEX T Verpoort et al, 2003 VIII European ISBT Congress P245



# PREPARATION OF METHYLENE BLUE –TREATED PLASMA UNDER WORST-CASE CONDITIONS - INFLUENCE ON QUALITY AND STABILITY-

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DGTI Congress, Erfurt, 2005

## INTRODUCTION

Treatment with methylene blue (MB) and light is a well-known procedure for the inactivation of blood-borne viruses in Fresh Frozen Plasma (FFP). The purpose of this study was to assess the quality and stability of MB/light-treated plasma (MB plasma) processed by the MacoPharma Theraflex MB-Plasma<sup>®</sup> system. Preparation was done under worst case conditions for routine processing to evaluate the worst plasma quality to be expected during production.

## METHODS

12 single donor units of MB/light treated plasma were prepared using the MacoPharma Theraflex MB-Plasma<sup>®</sup> system. Preparation included leukocyte depletion (Plasmaflex-filter), addition of methylene blue (MB-pill) prior to illumination and depletion of MB and photoproducts (Blueflex-filter) after treatment. Samples were taken before treatment and from the final product. For the assessment of stability, plasma from four different plasma pools was photodynamically treated and stored for up to 9 months. Treatment was done under worst-case conditions for the preservation of product quality: maximum MB concentration during illumination (1.15 µmol/l), maximum storage time of whole blood before separation (4°C, 17 h), maximum storage time of MB plasma before freezing (1 h).

## RESULTS

Thrombin time, fibrinogen (Claus), factors V, VIII, XI and protein C were significantly altered by MB/light treatment, while anti-thrombin III (AT III), vWF:RCo, vWF cleaving protease (vWF CP), plasmin inhibitor and  $\alpha_1$ -antitrypsin remained unchanged (Fig. 1). There was no activation of the coagulation markers (F 1+2, D-dimers) attributed to the virus inactivation procedure including the filtration steps for leukocyte depletion and MB and photoproduct depletion. The influence of each manufacturing step on the activity of coagulation factors was investigated using three plasma pools. Most of the activity was lost during illumination (Fig. 2). After illumination MB and its photoproducts (azure A, azure B, azure C) were depleted by Blueflex filtration (Fig 3) to a final concentration of <0.1 µmol/l (MB + sum of photoproducts). Stability of MB-Plasma was tested during storage at -30°C for up to 9 months (Fig 4). Stability testing will be continued for a total of 27 months.

Parameter		Before treatment	After treatment	Percentage of loss (-) or increase (+)
Thrombin time	[s]	15.8 +/- 0.7	19.1 +/- 1.5	+20.6 †
Fibrinogen (Claus)	[mg/dl]	279.9 +/- 53.6	222.5 +/- 41.8	-20.3 †
Factor V	[%]	120.7 +/- 30	101.8 +/- 29.8	-16.4 †
Factor VIII	[%]	115.2 +/- 23.9	89.5 +/- 21.3	-22.2 †
Factor XI	[%]	94.6 +/- 20.1	82.1 +/- 18.7	-13.3 †
Antithrombin III	[%]	87.6 +/- 7.4	87.3 +/- 6.0	-0.3 ns
Protein C	[%]	105.3 +/- 21.3	94.5 +/- 17.4	-9.8 †
Protein S, free	[%]	94.2 +/- 11.2	94 +/- 12	-0.2 ns
vWF:RCo	[%]	98.8 +/- 23.5	98.8 +/- 23.5	-5.0 ns
vWF CP	[%]	52.8 +/- 13.9	50.2 +/- 9.7	-1.8 ns
Plasmin inhibitor	[%]	94.2 +/- 9.1	93 +/- 8.7	-1.2 ns
$\alpha_1$ -Antitrypsin	[mg/dl]	95.8 +/- 19.3	95.3 +/- 19	-0.5 ns
F 1+2	[nmol/l]	1.02 +/- 0.25	1.03 +/- 0.27	+1.0 ns
D-Dimers	[mg/l]	0.20 +/- 0.04	0.18 +/- 0.04	-9.2 ns
CH 100	[U/ml]	618 +/- 115	633 +/- 169	+4.1 ns

n = 12, † significant, P < 0.01 (Wilcoxon Rank Sum Test) ns not statistically significant

Fig.1 Influence of the MB/light treatment on plasma quality (data from 12 single donor units, treatment under worst-case conditions)

Methylene Blue	µmol/l	Depletion (C / A)
A (before illumination)	0.97 +/- 0.06	
B (after illumination)	0.65 +/- 0.15	
C (after Blueflex filtration)	0.01 +/- 0.01	98.8 %

Azure A	µmol/l
A	0.01 +/- 0.01
B	0.06 +/- 0.03
C	0.01 +/- 0.01

Azure B	µmol/l
A	0.11 +/- 0.01
B	0.24 +/- 0.04
C	0.01 +/- 0.01

Azure C	µmol/l
A	0.00 +/- 0.00
B	0.03 +/- 0.03
C	0.01 +/- 0.02

Phenothiazine total	µmol/l	Depletion (C / A)
A	1.09 +/- 0.07	
B	0.99 +/- 0.08	
C	0.04 +/- 0.03	96.4 %

Fig.3 Depletion efficacy of the Blueflex filter (data from 12 single donor units)

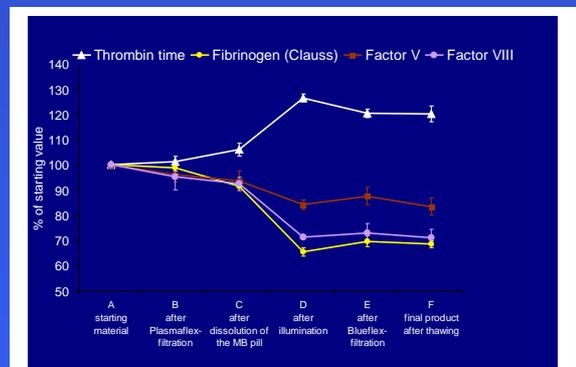


Fig.2 Influence of the individual manufacturing steps on plasma quality (data from three pools)

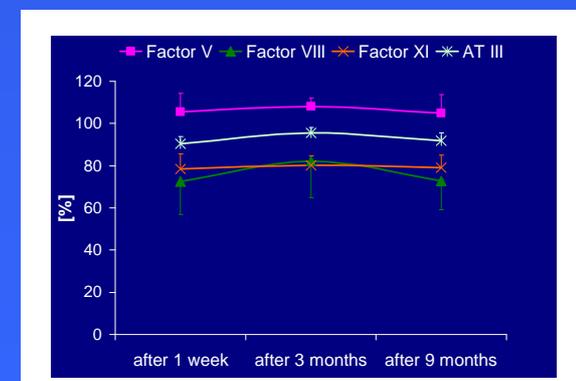


Fig.4 Stability of MB/light-treated plasma (data from 4 pools, treatment under worst-case conditions)

## CONCLUSIONS

Even under worst-case conditions, photodynamic treatment of FFP using the Theraflex MB-Plasma<sup>®</sup> system only moderately affects the activities of coagulation factors. The Blueflex-filter depletes MB and its photoproducts by over 90% after photodynamic treatment. Storage of MB plasma for up to 9 months had no effect on coagulation factors.



# THERAFLEX – MB PLASMA

## Coagulation factors and activation parameters

Parameter	Limits	For each stage in the preparation						
		Samp.A (n = 40)	Samp.B (n = 40)	Samp.C (n = 10)	Samp.D (n = 10)	Samp.A (n = 10)	Samp.Ab (n = 10)	Samp.B (n = 10)
Prothrombin rate (%)	70 - 130	80.8	71.8	87.7	76.6	73.5	68.2	65.7
INR	1	1.21	1.33	1.11	1.24	1.3	1.39	1.43
Activated partial thromboplastin time test (ratio)		1.12	1.26	1.05	1.18	1.13	1.22	1.27
Fibrinogen	2 - 4	3.11	2.29	2.94	2.04	3.03	2.28	2.36
Factor II (%)	70 - 120	102	98	98	95.1	95.1	86.7	92.3
Factor V (%)	70 - 120	102.2	94.1	89.5	83.2	105.7	98.1	96.4
Factor VII (%)	70 - 130	113.1	101.9	106.1	92.1	107.6	100.6	104.2
Factor VIII (%)	60 - 150	100.6	73.5	101.1	76.1	114	89.5	86.1
Factor IX (%)	60 - 150	96.5	78.8	98.6	86	99.2	78.9	85.7
Factor X (%)	70 - 120	105.9	95.3	106.9	92.6	97.3	91.7	92.3
Factor XI (%)	60 - 140	90.9	75.3	86.7	64.4	85.5	70.8	65.7
Factor XII (%)	60 - 140	103.7	92.6	110.5	99.9	98.4	96	93.9
Antithrombin III (%)	80 - 120	104.8	89.9	104.4	101.9	105.8	103.7	93.9
Protein C (%)	70 - 140	110.9	105.3	108.4	103	120.7	112.9	112.6
Protein S (%)	70 - 140	82.6	78.6	81.6	71.2	83.8	77.6	81.4
V Willebrand Factor CoF ristocetin (%)	60 - 150	97.6	92.9	87.4	84	143.6	137.4	138.6
Von Willebrand Factor Ag (%)	60 - 150	134.6	118.2	130.3	128	143.6	121.8	122.2
Plasminogen (%)	80 - 120	102.7	100.9	100.8	96.9	103.6	101.6	101.6
$\alpha$ 2-antiplasmin (%)	80 - 120	111.9	107.7	106.2	104.2	112.5	106.5	107.7
C3a (mg/l)	100 - 400	134.8	134.1	146.8	143.1	124.2	125.5	124.0
C5a ( $\mu$ g/l)	0.9 - 15.4	10.9	14.1	15.1	23.9	7.5	8.3	10.9
Factor XIIIa (ng/ml)	< 3.0	2.39	2.22	2.87	2.63	2.57	1.79	2.49
F1 + 2 prothrombin (nmol/l)	0.4 - 1.1	0.99	1.20	0.88	0.87	0.79	0.72	0.78
Platelet factor 4 (UI/ml)	56 - 805	317.2	301.1	413.9	375.4	186.9	197.2	208

A : Plasma before contact with methylene blue

Ab : Plasma after visible light (plasmas 31 to 40)

B : MB-removed Plasma before freezing

C : MB Plasma after 6 months at - 30° C

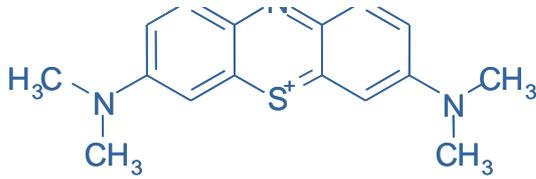
D : MB-removed Plasma after 6 months at - 30° C



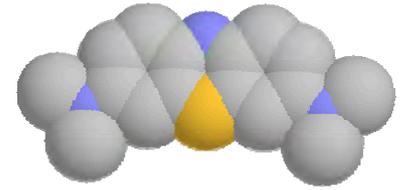


# THERAFLEX-MB Plasma

## Processing principle



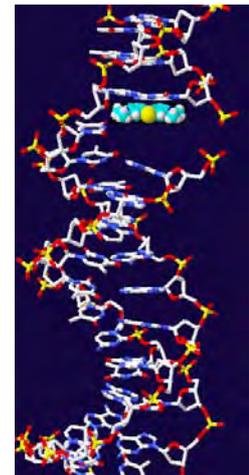
Methylene Blue molecule



**MacoPharma** Methylene Blue Pill  
(85µg / unit of plasma)

Illumination of plasma + Methylene Blue  
(590 nm, 180J/cm<sup>2</sup>)

- Intercalation of MB into nucleic acids
- Excitation of MB by visible light
- Oxidation of Guanosine
- Degradation of nucleic acids



The combined action of Methylene Blue and light is a photodynamic process which blocks transcription and replication of viral RNA and DNA.

### Photo-inactivation procedure of THERAFLEX-MB PLASMA

200-315ml of plasma  
(Aphaeresis or Whole Blood)

Filtration of plasma and dissolution of the MB Pill

Illumination of plasma+MB with the Macotronic V4

MB removal by filtration with Blueflex

Plasma freezing





# THERAFLEX-MB Plasma

## Examples of systems and treatments

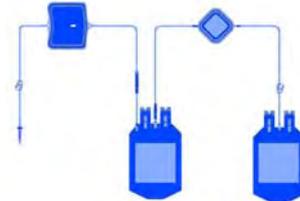
### Whole Blood Plasma with MB treatment



**BSV system** : plasma filtration with Plasmaflex  
> MB Pill dissolution > illumination

Whole blood plasma  
Min 250mL - max 330 mL\*

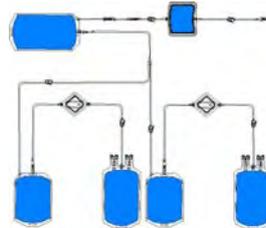
### Whole Blood Plasma with MB treatment and MB removal



**SDV system** : plasma filtration with Plasmaflex  
> MB Pill dissolution > illumination > MB removal with Blueflex

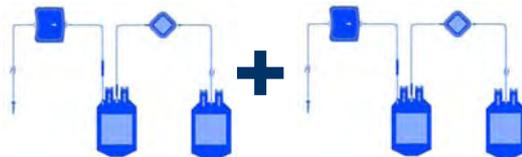
### Aphaeresis Plasma : distribution in 2 units

Aphaeresis plasma  
Min 485 mL – max 650mL\*



**SPV system** : filtration of the aphaeresis unit with Plasmaflex MB Pill dissolution and distribution in 2 units > illumination > MB removal with Blueflex

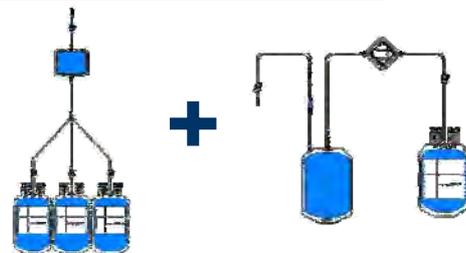
Aphaeresis plasma  
Min 500 mL – max 665mL\*



**SDV system** : plasma filtration with Plasmaflex > MB Pill dissolution > illumination > MB removal with Blueflex

### Aphaeresis Plasma : distribution in 3 units

Aphaeresis plasma  
> 690 mL\*



**BSV + ZDV system** : filtration of the aphaeresis unit with Plasmaflex and distribution in 3 units with the BSV system. Connection of each unit to a ZDV system : MB Pill dissolution > illumination > MB removal with Blueflex

\* Volume ranges for plasmas to be treated with THERAFLEX-MB Plasma, based on process requirements.

## Methylene blue-treated fresh-frozen plasma: what is its contribution to blood safety?

*Lorna M. Williamson, Rebecca Cardigan, and Chris V. Prowse*

**W**ith current donor-selection criteria and virus genome testing, fresh-frozen plasma (FFP) in the developed world is probably safer than it ever has been. In the UK, where FFP is not manufactured from first-time or lapsed donors, it has been estimated that the residual virus 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 (Eglin R, written communication, January 2003). Against these levels of risk, it has been questioned whether pathogen reduction of FFP is a necessary strategy and/or the best use of healthcare resources.<sup>1</sup> However, the appearance of West Nile virus in blood components in the US in 2002, with fatal transmissions in immunocompromised recipients,<sup>2</sup> reminds us that sometimes viruses move ahead of our ability to test for them. Also, background viral incidence in a population can change, as is currently observed in Scotland, with HIV levels showing an increase to three per million population (Soldan K, written communication, February 2003). It is now over 10 years since a photodynamic system using methylene blue (MB) and visible light was developed in Springe, Germany, for virucidal treatment of FFP. The method has been used at various times since then in Germany, Denmark, Portugal, Spain, and the UK, so it is timely to review its potential contribution to overall FFP safety.

MB is a phenothiazine compound (Fig. 1), which was first used clinically by Paul Ehrlich in the 1890s and has been used to kill viruses since work at the Walter Reed

Hospital in the 1950s.<sup>3</sup> When activated by visible light, MB generates reactive oxygen species, mainly singlet oxygen, through a Type II photodynamic reaction, and it is these that are responsible for its pathogen inactivating properties.<sup>3-5</sup> The original system developed in Springe, Germany, used an initial freeze-thaw step to disrupt intact WBCs, then added an amount of MB solution calibrated to the weight of the plasma pack, to achieve precisely the same MB concentration in every pack. Later systems (Baxter and Macopharma) developed for small-scale use in blood centers involve sterile connection of the plasma pack (before or after freezing) to a pack with a WBC-reduction filter upstream of a liquid pouch or a dry pellet containing 85 to 95 µg of MB (Fig. 2). To achieve the desired final MB concentration of 1 µM, the input plasma volume has to be within a 200-to-300-mL range, so 600-mL apheresis units require splitting. In both the Springe and commercial systems, the MBFFP packs are then exposed to visible wavelengths of light to activate the MB. Because it is not possible to use the equivalent of radiation-sensitive labels to confirm illumination, the light-exposure system must be designed to ensure good manufacturing practice (GMP)-compliant control of both light intensity and duration. Radio-frequency chips for this purpose are in development. During illumination, MB is converted to its bleached leuco- form and to demethylated components (azure A, B, and C, and thionine; Fig. 1). A recent feature has been the development of commercial filters for post-treatment MB removal, which reduce the residual MB concentration to 0.1 to 0.3 µM. The plasma is then ready for freezing or refreezing.

One of the attractions of the technique is that it is applied to single units of FFP, without the need for pooling. Commercial systems are available that can be set up in standard blood center GMP conditions, without the need to install specialized plant, and it is this model that is in operation in the UK. Plasma is frozen locally, sent to one of three central MB-treatment points, then returned for distribution to hospitals.

### PATHOGEN-REDUCTION SPECTRUM

The ability of MB to inactivate viruses is dependent on its binding to nucleic acid, being greater for double stranded

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**ABBREVIATIONS:** APTT = activated partial thromboplastin time; FFP = fresh-frozen plasma; MB = methylene blue; MBFFP = methylene blue-treated fresh-frozen plasma; PT = prothrombin time; TTP = thrombotic thrombocytopenic purpura.

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than single stranded, although viruses containing genomes of either type may be efficiently inactivated (see below). Activation results in a mixture of strand cross-linking, guanosine oxidation, and depurination. MB may also modify proteins and lipids, the relative rates depending on the MB and local oxygen concentrations. For virus-infected cells, this may be influenced by the reducing and detoxifying mechanisms present inside the cell. MB is not considered useful for inactivation of intracellular viruses or to attain bacterial or protozoal reduction, although it does enter cells.<sup>5-7</sup> Its only application in transfusion has been to achieve virus inactivation of plasma, with prior cell removal by filtration or freeze-thaw lysis<sup>8-10</sup> (Flament J, Mohr H, and Walker W, written communication, 2000).

Photodynamic treatment with MB results in efficient virus inactivation for all lipid-enveloped viruses tested to date, including all those for which the UK and US currently routinely screen blood donations, as well as West Nile virus.<sup>3-5,10</sup> The extent of removal for such viruses is usually at least 5 logs, this being true for both double- and single- stranded RNA and DNA viruses (Table 1). Nonlipid-enveloped viruses show a more diverse spectrum of susceptibility, some being totally unaffected (EMC, polio, HAV, porcine parvovirus), whereas others (SV40, HEV models, human parvovirus B19) show reduction factors of 4 logs or more (Table 1). More recently, testing using PCR methods has shown direct removal of HIV, HBV, HCV, and parvovirus B19 reactivity from infected donations,<sup>11-14</sup> the last of these demonstrating 4-log reduction by a newly developed B19 bioassay on the KU 812 EP 6 cell line (Flament J, Mohr H, and Walker W, written communication, 2000).

Are such reduction factors sufficient to assure that a single plasma donation, taken during the peak of viremia, is rendered noninfectious? The answer will depend on whether the donation is also subjected to NAT or serologic testing and on the level of viremia. For most viruses, we know that the answer is almost certainly yes, but in a few cases such as parvovirus B19, in which the peak of viremia is around  $10^7$  genome equivalents per mL, this conclusion is more dubious. However, for viruses of major concern, peak viremia levels are either within the clearance range of the system, or screening with assays of high sensitivity

## StainsFile *Methylene blue homologues*

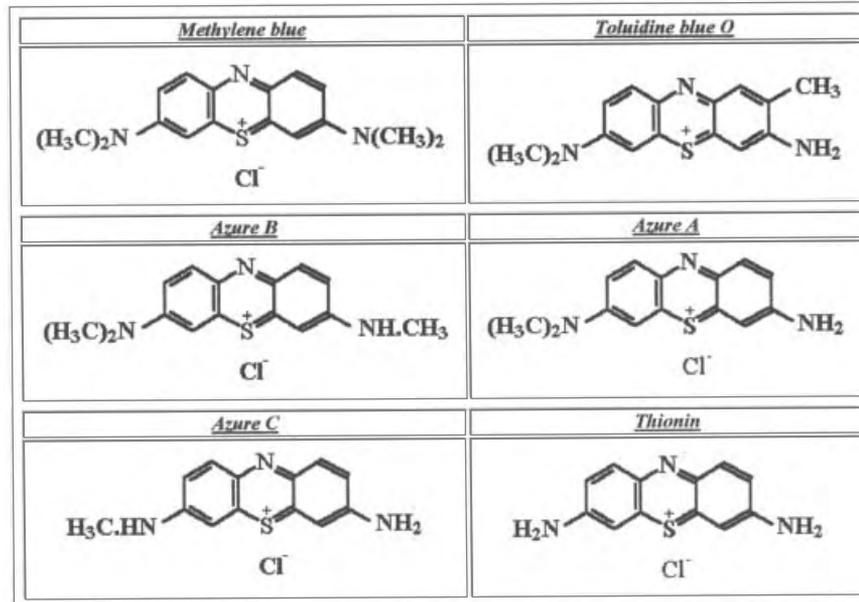


Fig. 1. MB and its photodegradation products.

TABLE 1. Virus reduction			
Lipid enveloped		Non-lipid enveloped	
Virus	log reduction factor	Virus	log reduction factor
HIV	>5.5	HAV	0.0
Bovine viral diarrhea	>6.2	Encephalomyocarditis	0.0
Duck HBV	3.9	Porcine parvovirus	0.0
Influenza	5.1	Polio	0.0
Pseudorabies	5.4	SV40	4.3
Herpes simplex	>6.5	Adenovirus	4.0
Vesicular stomatitis	>4.9	Human parvovirus B19	≥4.0
West Nile virus	>6.5	Calicivirus (HEV)	>3.9

will have ensured that only donations with lower levels of viremia enter the processing laboratory (handling errors excepted). In the pregenome testing era, there was a possible HCV exposure from a unit of MBFFP taken from a donor in the sero-negative window period (Flament J, written communication, March 1998). The patient seroconverted for HCV but remained genome negative. The precise events remain unproven, but it is possible that the patient generated an antibody response against inactivated virus.

Although MB and other phenothiazine dyes have been suggested as having inhibitory action against transmissible spongiform encephalopathies,<sup>15</sup> there is no evidence of in vitro inactivation of infectivity at the concentrations used in the transfusion setting.

## The MACO PHARMA Plasma Membrane filtration Methylene Blue Illumination and MB Depletion Set

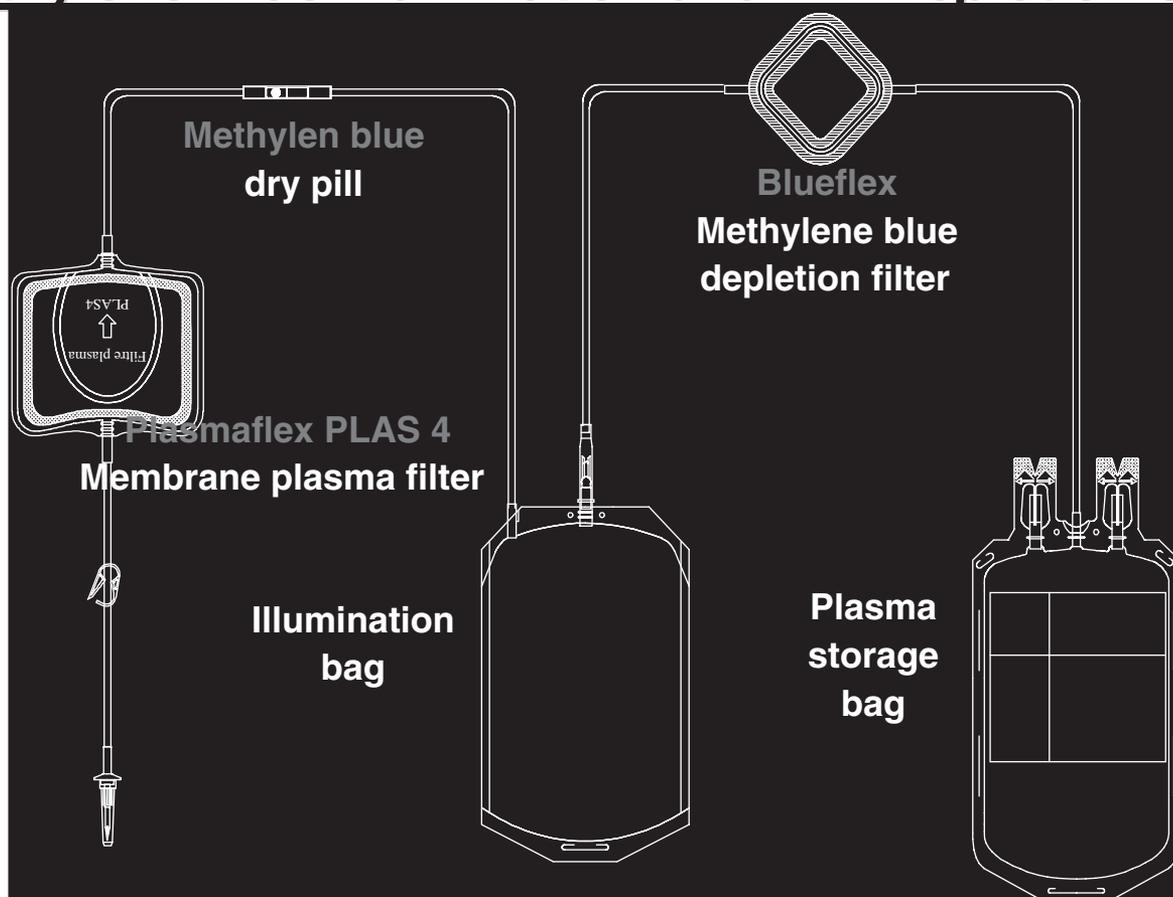


Fig. 2. Schematic representation of the closed bag system for MB treatment of fresh-frozen plasma.

### EFFECT OF MB TREATMENT ON COAGULATION PROTEINS

It is well established that MB treatment of plasma affects the functional activity of various coagulation proteins and inhibitors (Table 2). The proteins most severely affected by MB treatment of plasma are FVIII and fibrinogen, where activity is reduced by 20 to 35 percent. The decrease in fibrinogen is seen when assayed by the method of Clauss, but not in antigenic assays,<sup>16</sup> suggesting that MB treatment effects the biologic activity but not concentration of fibrinogen. It has been suggested that this is due to the photo-oxidation of fibrinogen inhibiting polymerization of fibrin monomers.<sup>17</sup> The effects on fibrinogen are probably due to an interaction of MB with histidine residues and may result in a modified *in vivo* clearance.<sup>16,18-20</sup> However, fibrinogen isolated from MB-treated plasma retains normal ability to bind to glycoprotein IIb/IIIa receptors on platelets,<sup>21</sup> an important mechanism in platelet activation

and aggregation. The inhibitory effects are ameliorated by the presence of ascorbate<sup>22</sup> but do not appear to result in the formation of any neoantigens<sup>16,18,19</sup> or positivity in tests for the formation of IgE antibodies (Flament J, Mohr H, and Walker W, personal communication, 2000).

Unsurprisingly, the changes in coagulation proteins observed in MB-treated plasma are associated with a prolongation of the prothrombin time (PT) and activated partial thromboplastin time (APTT).<sup>16,23</sup>

Original studies on MB inactivation were reported on plasma freeze-thawed before treatment, but later work on the Baxter Pathinact and Maco Pharma Theraflex systems was performed on fresh plasma (Table 2). However, we have recently shown that the major cause of coagulation factor loss is the MB treatment itself and not the freeze-thawing.<sup>16,24</sup> Fortunately, changes in coagulation proteins induced by WBC-reduction and MB-removal filters appear to be negligible compared to the effect of the MB process itself. Filtration of plasma using a filter (Hemasure)

TABLE 2. Changes in coagulation factor proteins and inhibitors in MBFFP

Parameter*	Percent change due to MB treatment†‡§	Mean residual levels‡§
Fibrinogen (Clauss) g/L	↓ 24, <sup>10</sup> 24, <sup>23</sup> 39 <sup>29</sup>	1.65, <sup>10</sup> 1.80, <sup>16</sup> 2.01, <sup>23</sup> 1.97, <sup>28</sup> 2.05 <sup>29</sup>
Fibrinogen (antigen) g/L		2.74 <sup>16</sup>
Prothrombin (FII) (U/mL)	↓ 8, <sup>10</sup> 8, <sup>16</sup> 18, <sup>23</sup>	1.15, <sup>10</sup> 1.05, <sup>16</sup> 1.00 <sup>23</sup>
FV (U/mL)	↓ 4.5, <sup>10</sup> 21, <sup>16</sup> 32, <sup>23</sup> 10, <sup>28</sup>	0.84, <sup>10</sup> 0.73, <sup>16</sup> 0.79, <sup>23</sup> 0.76 <sup>28</sup>
FVII (U/mL)	↓ 8, <sup>10</sup> 9, <sup>16</sup> 7, <sup>23</sup>	1.10, <sup>10</sup> 0.90, <sup>16</sup> 0.90 <sup>23</sup>
FVIII (U/mL)	↓ 13, <sup>10</sup> 33, <sup>16</sup> 28, <sup>23</sup> 26, <sup>28</sup> 29 <sup>29</sup>	0.78, <sup>10</sup> 0.58, <sup>16</sup> 0.58, <sup>23</sup> 0.83 <sup>28</sup>
FIX (U/mL)	↓ 17, <sup>10</sup> 23, <sup>23</sup> 11 <sup>28</sup>	1.00, <sup>10</sup> 0.72, <sup>23</sup> 0.88 <sup>28</sup>
FX (U/mL)	↓ 13, <sup>10</sup> 7 <sup>23</sup>	1.05, <sup>10</sup> 0.90 <sup>23</sup>
FXI (U/mL)	↓ 17, <sup>10</sup> 27, <sup>23</sup> 13 <sup>28</sup>	1.00, <sup>10</sup> 0.73, <sup>23</sup> 0.84 <sup>28</sup>
FXII (U/mL)	↓ 17 <sup>10</sup>	1.20 <sup>10</sup>
FXIII (U/mL)	↓ 7, <sup>23</sup> 16 <sup>29</sup>	1.02, <sup>23</sup> 1.12 <sup>29</sup>
vWF antigen (U/mL)	↓ 7, <sup>23</sup> 5 <sup>29</sup> → <sup>28</sup>	0.94, <sup>23</sup> 0.83, <sup>29</sup> 1.00 <sup>28</sup>
vWF:ristocetin cofactor(U/mL)	↓ 8, <sup>23</sup> 18 <sup>29</sup>	0.92, <sup>23</sup> 0.79 <sup>29</sup>
C1-inhibitor (U/mL)	↓ 23, <sup>10</sup> → <sup>16</sup>	0.88, <sup>10</sup> 1.03 <sup>16</sup>
Antithrombin (U/mL)	↓ 8, <sup>10</sup> 3 <sup>23</sup> → <sup>16,23</sup>	0.78, <sup>10</sup> 0.95, <sup>16</sup> 1.00, <sup>23</sup> 0.96 <sup>28</sup>
Protein C (U/mL)	→ <sup>16,28</sup>	1.03, <sup>16</sup> 0.89 <sup>28</sup>
Protein S (U/mL)	→ <sup>16</sup>	1.11 <sup>16</sup>
α <sub>1</sub> -antitrypsin (U/mL)	→ <sup>16</sup>	155 mg/dL
Plasminogen (U/mL)	→ <sup>10,16</sup>	0.90, <sup>10</sup> 0.98 <sup>16</sup>
α <sub>2</sub> -antiplasmin (U/mL)	→ <sup>16</sup>	0.96 <sup>16</sup>

\* Results given as U/mL because not all studies were calibrated against international standards. Assays are functional unless otherwise stated.

† Arrows indicate direction of change, with horizontal arrow indicating no change.

‡ <sup>10,16,23</sup> Studies used frozen-thawed plasma.

§ <sup>28,29</sup> Studies used fresh plasma (<8 hr from collection).

designed to remove both WBCs and MB simultaneously results in a prolongation of the APTT but has no effect on the PT or fibrinogen when measured by manual techniques.<sup>25</sup> Filters to remove residual MB in plasma developed more recently by Pall and Maco Pharma are reported to result in a small increase in the APTT but minimal loss of coagulation factor activity.<sup>26,27</sup> It has been suggested that the increase in the APTT in the latter studies may be a result of some activation of the contact system of coagulation after contact of plasma with the artificial surface of the filter.<sup>26</sup>

Levels of thrombin-antithrombin complexes are not elevated in MB-treated plasma,<sup>16</sup> indicating that MB treatment is also not associated with excessive thrombin generation. Functional measurements of the naturally occurring anticoagulants protein C & S and antithrombin also appear to be relatively unaltered in MB-treated plasma.<sup>10,16,23,28</sup> MB treatment is reported to have little effect on levels of plasminogen, alpha-2-antiplasmin (the main inhibitor of plasmin), fibrin monomer, and D-dimers,<sup>16</sup> suggesting that the use of MBFFP is unlikely to result in enhanced fibrinolysis. vWF activity in plasma, as measured by ristocetin-induced agglutination of platelets, is reduced by 10 to 20 percent,<sup>23,29</sup> but vWF multimeric distribution and cleaving protease activity are reported to be unaffected.<sup>23,28-30</sup>

After transfusion of MB-treated plasma to healthy adults, there was no significant difference from baseline values in APTT, PT, TT, FVIII, FXI, Clauss fibrinogen, fibrin degradation components, or platelet aggregation induced

by collagen or ADP, suggesting no major influence on coagulation or fibrinolytic systems.<sup>31</sup>

There have been relatively few studies examining cryoprecipitate and cryosupernatant produced from MB plasma. Levels of FVIII and fibrinogen activity in cryoprecipitate are 20 to 40 percent lower than untreated units,<sup>23,32</sup> but remain within Council of Europe Guidelines. The effect on levels of vWF antigen and activity seem more variable: one study reports no significant difference,<sup>23</sup> whereas in a two-center study, one center also reported no change, while the other saw 15 to 20 percent lower values in MB units.<sup>32</sup> These differences might be explained by variation in the methodology used to prepare the cryoprecipitate. However, both studies show that the multimeric distribution of vWF is unaltered. Cryoprecipitate produced from MBFFP has not yet been introduced in any country that provides MBFFP, but work is ongoing in the UK to optimize fibrinogen concentration.<sup>33</sup>

Cryosupernatant produced from standard or MB-treated plasma lacks the largest molecular weight forms of vWF.<sup>23</sup> The main clinical indication for cryosupernatant is for the treatment of thrombotic thrombocytopenic purpura (TTP). Patients with TTP tend to have unusually large molecular weight vWF multimers,<sup>34</sup> which are known to promote platelet aggregation, and some believe that treatment with a plasma component that lacks the high molecular weight forms of vWF may be beneficial. However, no clinical data are available to answer this question. Levels of vWF cleaving protease have not been measured in cryosupernatant produced from MB-treated plasma, but given

that levels appear to be relatively unaltered in the source plasma,<sup>30</sup> one would not expect them to differ significantly. It would thus appear that MB-treated cryosupernatant would be suitable for the treatment of TTP, but it has yet not been manufactured for clinical use.

If MB plasma is used to suspend single-donor platelets, there is no significant effect on platelet numbers, morphology scores, osmotic recovery, or levels of LDH, CD62P expression, lactate, pH, and glucose compared to standard plasma.<sup>35</sup> Similarly, if MB-treated plasma is added to RBCs, there appears to be no appreciable effect on leakage of potassium, hemolysis, or osmotic fragility during 28 days of storage.<sup>35</sup> This is in contrast to direct treatment of RBCs with MB and light, which results in membrane leakage and enhanced surface binding of IgG.<sup>5-7</sup>

## PHARMACOLOGY AND TOXICOLOGY

The major clinical application of MB in the past has been as a redox reagent in the reversal of methemoglobinemia and cyanide poisoning using intravenous doses of 1 to 5 mg per kg. It has also been used at higher oral doses for the treatment of manic depression (300 mg/day) and renal calculus disease (195 mg/day). Intravenous doses of 2 to 5 mg per kg have also been used for heparin neutralization and for perioperative staining of the parathyroid gland.<sup>3,4,9,10,20</sup> For comparison, the plasma pathogen-reduction systems described here result in a MB concentration of 1  $\mu\text{M}$  in the FFP, equivalent to an intravenous dose per 250 mL FFP unit of 0.0012 mg per kg. If MB-removal filters are used during processing,<sup>25,36</sup> this level is reduced approximately  $\times 10$ , to a final concentration of 0.1 to 0.3  $\mu\text{M}$ . For a 70-kg adult receiving the recommended 15 mL per kg of FFP, this equates to a total MB dose of approximately 33  $\mu\text{g}$ , or less than 1  $\mu\text{g}$  in a 2-kg premature infant. Infused MB is rapidly cleared from the circulation and marrow (half-lives in rats are 7 and 18 min, respectively) to an extent that its presence in blood (half-life in man approx. 60 min) is difficult to detect after infusion of MBFFP. There is some tissue uptake, but the majority of MB is excreted via the gastrointestinal tract and in urine within 2 or 3 days<sup>3</sup> (Flament J, Mohr H, and Walker W, written communication, 2000).

A US toxicologic report summarizes its use to assess membrane rupture during amniocentesis, noting mild and transient side effects at most.<sup>37</sup> In mammals, the half lethal dose for MB is of the order of 100 mg per kg, with photo-illumination products having similar, or lesser, toxicity profiles to the parent compound.<sup>35</sup> Chronic dosing of animals with MB at doses up to 0.2 g per kg day for 13 weeks are nontoxic. Chronic exposure of rats to a diet containing 4 percent MB had no carcinogenic or cirrhotic effects, while testing in both rodents and *Drosophila* revealed no genotoxic effects at near lethal doses. Testing

for induction of birth defects at doses up to 5 mg per kg per day has also given negative results,<sup>3,5</sup> although recently higher doses have been reported as inducing fetal growth retardation.<sup>38</sup> In contrast to this, *in vitro* tests, such as the Ames test for mutagenic effect in selected bacteria, have yielded some mutagenic and genotoxic data, particularly in the presence of a liver microsomal (S9) fraction. Testing on human lymphocytes and the mammalian V79 cell line has been reported by some to show no mutagenicity, although in the presence of the microsomal S9 fraction, some chromosomal aberrations were seen in lymphocytes at 1 to 2  $\mu\text{g}$  per mL (Flament J, Mohr H, and Walker W, written communication, 2000). Wagner et al.<sup>39</sup> has reported genotoxic effects in mouse lymphoma cells at 30  $\mu\text{g}$  per mL of MB, which was enhanced by S9 addition, but failed to detect any activity *in vivo* in a mouse micro-nucleus assay.

Between 1992 and 1998, more than a million units of MBFFP were used in Germany, Switzerland, Austria, and Denmark. Use has continued in the UK, Portugal, and Spain using the Grifols, Baxter, and Macopharma versions of the technology. The latter two systems have a European Medical Devices licence (CE mark), granting of which includes a toxicologic assessment. Both passive and active surveillance<sup>40</sup> have yielded adverse event rates that do not differ from those for standard FFP. In neonates, where the concern is greater due to the immature detoxification system, there are few reports on surveillance, but data from both Germany and Spain indicate no acute adverse events, even when MBFFP is used for exchange transfusion (Castrillo A, Pohl U, written communication, 1999). Concern over the potential *in vitro* mutagenic effects of MB and its derivatives, particularly in the presence of the S9 fraction, was the reason for the failure to re-license the product (without MB removal) in Germany in 1998. An opinion has not been reached on whether the system including the MB-removal step will be granted a German license. However, a large amount of clinical usage and *in vivo* toxicology testing suggest that despite the effects seen *in vitro*, *in vivo* side effects are minimal, presumably mainly due to the dilution on infusion and the rapid clearance of the compound. One toxicology expert in the field has suggested the risk is on a par with smoking a pack of cigarettes over a lifetime (Flament J, Mohr H, and Walker W, written communication, 2000).

## CLINICAL STUDIES

Most studies in patients have been small and/or have used laboratory rather than clinical endpoints. Despite usage of more than 1 million units in Europe, there have been no full reports of large, randomized trials of MBFFP using relevant endpoints such as blood loss or exposure to other blood components. Early studies described successful use of MBFFP in either single or small groups of patients with

deficiencies of FV or FXI, TTP, and exchange transfusion in neonates.<sup>41,42</sup> One study of 71 patients compared MBFFP with S/D-treated FFP in cardiac surgery and showed better replacement of protein S and alpha<sub>2</sub>-antiplasmin with MBFFP but no difference in blood loss.<sup>20</sup> However, one hospital in Spain has reported that after a total switch to MBFFP, FFP demand rose by 56 percent, with a two- to three-fold increase in demand for cryoprecipitate, which was not MB treated.<sup>43</sup> The authors suggest that the increase in demand, particularly for cryoprecipitate, may have been required to offset the reduced fibrinogen level in the component. Indeed, after orthopedic surgery, transfusion of MBFFP has been associated with increased reptilase clotting times and ratio of immunologic to functional measured fibrinogen,<sup>44</sup> suggesting that MB may interfere with fibrin polymerization *in vivo*. However, the data from the Spanish study need to be interpreted with care. In the period studied, which spanned introduction of MBFFP, 2967 patients received no fewer than 27,434 units of plasma, but only 24,607 units of RBCs, with 26 percent of admissions receiving FFP only. The very high FFP to RBC ratio (1.11) contrasts sharply with the recent corresponding figure for the UK Transfusion Services (0.14).<sup>45</sup> This suggests very different prescribing practices for FFP between Spain and the UK, including routine use of FFP in all cardiac surgery procedures in Spain.<sup>43</sup> Nevertheless, their study emphasizes the importance of monitoring clinical demand after any change to MBFFP, to see whether the *in vitro* effects truly result in a requirement for larger doses.

No specific data are available from studies in neonates, but no specific problems have been found. The only report of MB toxicity in a neonate was a case of severe bullus formation and desquamation was reported in a baby who received phototherapy for hyperbilirubinemia after administration of 10 mL of 1 percent MB to the mother to investigate possible rupture of amniotic membranes.<sup>46</sup> Although neonatal blood levels of MB were not reported, the skin of the baby was visibly stained blue, suggesting blood and tissue levels many times higher than would be achieved after infusion of MBFFP. No problems with MBFFP-treated infants requiring phototherapy have been reported in Europe, and glucose 6 phosphate dehydrogenase deficiency is not a contra-indication to its use (Walker W, written communication, 2002). Similarly, digital capillary measurement of oxygen saturation by colorimetric means is not affected by infusion of MBFFP.

Limited data are available on the use of MBFFP for plasma-exchange procedures for TTP.<sup>47</sup> Although levels of vWF cleaving enzyme in MBFFP are normal,<sup>30</sup> one study of two small cohorts of patients (13 treated with FFP and 7 with MBFFP) reported an increase in the number of plasma-exchange procedures and days in hospital in the MBFFP group.<sup>48</sup> This is of concern, although the small patient numbers make it difficult to draw conclusions;

clearly, larger studies are required to establish the role of MBFFP in TTP.

### FFP SAFETY: WHERE ARE WE GOING?

Five years ago, an editorial in this journal accompanied the availability in the USA of pooled S/D FFP.<sup>1</sup> Despite the impact of the previous HIV and HCV transmissions on transfusion services in many countries, S/D FFP did not subsequently become a standard of care in the USA, although it has become so in Norway, Belgium and Portugal. Other European countries have chosen quarantining of FFP with donor re-test as their method of minimizing virus risk from FFP. This avoids potential toxicity or loss of activity, but provides no protection against new agents such as West Nile virus. In virus reduction terms, the MB system appears to have acceptable efficacy, and has the advantage of being a single unit system, so that potentially increased risks from new agents unaffected by the system, such as prions, are minimized. The major disadvantage is loss of coagulation factors, such as fibrinogen. The as yet unlicensed single unit psoralen S59 pathogen reduction system for FFP appears to result in much better preservation of fibrinogen, with only 3-13 percent reduction.<sup>49</sup> However, toxicity will be a concern for any pathogen reduction system which interacts with nucleic acids, especially if administered to very young recipients.

In the UK, provision of MBFFP is linked to the most recent Department of Health precautionary decision to minimize the unknown risk of variant CJD from UK blood components. In August 2002, UK Transfusion Services were instructed to seek supplies of US plasma for FFP production for children born after January 1, 1996, a date from which the UK food supply has been considered safe from bovine spongiform encephalopathy. This imported FFP will be subjected to MB treatment, and, in preparation, UK Transfusion Services have already introduced MBFFP for this age group. No immediate problems with side effects or loss of efficacy have been reported, although the number of children treated is still small. Hospitals also have access to S/D FFP from commercial sources.

But to take an overview of FFP safety, 5 years' hemovigilance data in the UK reveal that virus transmission is a much smaller risk than that of TRALI. From 1996 to 2001, there were 15 TRALI cases in which FFP was clearly implicated, and another 4 where FFP was among a range of components transfused. In the same time period, there was not a single proven virus transmission from FFP.<sup>45</sup> Single-unit pathogen-reduction systems by themselves contribute nothing to TRALI prevention, which may be helped by selection of male donors for FFP<sup>50</sup> and/or screening of parous females for WBC antibodies. Interestingly, the pooling of several hundred donations required in the S/D FFP process may provide benefit against TRALI

by diluting out those with high-titer WBC antibodies. The National Blood Service in England has begun a formal option appraisal of TRALI-prevention strategies, beginning with plasma-rich components. The relative cost effectiveness and long-term role of pathogen reduction of FFP in an overall blood safety strategy remain to be elucidated.

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## The effect of methylene blue photoinactivation and methylene blue removal on the quality of fresh-frozen plasma

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**BACKGROUND:** The effects of using fresh or frozen-thawed plasma, WBC reduction of plasma before freezing, and the use of two different methylene blue (MB) removal filters on the quality of MB-treated plasma were compared.

**STUDY DESIGN AND METHODS:** In a paired study (n = 11/arm) plasma was frozen within 8 hours of collection, thawed, MB photoinactivated, and then filtered using one of two MB removal filters. Fresh plasma (n = 16) and plasma WBC reduced before freezing (n = 19) were MB inactivated.

**RESULTS:** Freeze-thawing resulted in loss of activity of FXII and VWF of 0.06 and 0.04 units per mL, respectively, but no significant loss of activity of factors II through XI or fibrinogen. Further loss of activity occurred after MB treatment: FII (0.07 IU/mL), FV (0.11 U/mL), FVII (0.08 IU/mL), FVIII (0.28 IU/mL), F IX (0.12 IU/mL), FX (0.16 IU/mL), FXI (0.28 U/mL), FXII (0.15 U/mL), VWF antigen (0.05 IU/mL), VWF activity (0.06 U/mL), and fibrinogen (0.79 g/L). Losses due to this step were significantly (5-10%) lower in fresh plasma compared to frozen-thawed plasma. Neither MB removal filter resulted in significant loss of activity of any factor studied.

**CONCLUSION:** MB removal, by either of the available filters, has little impact on the coagulation factor content of plasma, but freezing of plasma before MB treatment results in a small additional loss.

Due to stringent donor selection and testing procedures, fresh-frozen plasma (FFP) in the developed world offers a high degree of viral safety. For example, the risk of an infectious FFP donation entering the blood supply in England is estimated to be 1 in 10 million for HIV, 1 in 50 million for HCV, and 1 in 1.2 million for HBV (Eglin R, written communication, 2002). Nevertheless, viral transmission from blood components continues to occur, with 16 cases reported in the UK in the last 6 years.<sup>1</sup> There is, therefore, considerable research activity in pathogen inactivation of single-unit components because methods suitable for single components offer reassurance that no increased infectious risks are added due to pooling. For FFP only, one licensed single-unit system is currently available (methylene blue photoinactivation). It is desirable that there is as much flexibility as possible in the handling conditions for plasma before inactivation, to enable production of FFP from collection centers distant from the processing site. This is particularly relevant because the UK Departments of Health have recently recommended that FFP is imported from North America for neonates and children born after 1995 (after the introduction of relevant food bans to limit BSE transmission) as a precautionary measure against vCJD transmission. Previous studies have

**ABBREVIATIONS:** APC = allophycocyanin-conjugated; APTT = activated partial thromboplastin time; FFP = fresh-frozen plasma; MB = methylene blue; PMN, = neutrophil; PRP = platelet-rich plasma; PT = prothrombin time; VWF:CB = VWF collagen-binding activity.

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demonstrated vCJD infectivity in plasma of rodents infected with prion diseases,<sup>2,3</sup> and a recent report describes interim results from a study that demonstrate transmissions of bovine spongiform encephalopathy (BSE) and scrapie between sheep by whole-blood transfusion.<sup>4</sup> Because background levels of virus marker positivity in the North American population are significantly higher than in the UK, it has been deemed sensible to subject imported plasma to a pathogen-inactivation step.

The methylene blue (MB) photoinactivation process for viral inactivation of human plasma has been well described<sup>5</sup> as has its effect on the loss of coagulation factor activity of plasma.<sup>5-9</sup> The original Springe MB process, also used by Grifols in Spain, described by Lambrecht,<sup>5</sup> used freeze-thawing of plasma before MB inactivation to expose intracellular viruses to the action of MB. However, recently, blood collection packs that integrate WBC reduction and MB addition before inactivation of plasma (Baxter Pathinact, Baxter Healthcare, Compton Newbury, Berkshire, UK, and Maco Pharma Theraflex, Middlesex, UK) remove the need to freeze-thaw plasma.<sup>10-12</sup> There are also differences between the systems in how MB is added to plasma. With two of the systems (Springe and Baxter), a variable dose of MB solution is added to achieve a standard final concentration of 1  $\mu\text{M}$  MB. The other system (Maco Pharma Theraflex) incorporates an 85- $\mu\text{g}$  pellet of MB hydrochloride per plasma unit, therefore the concentration can vary slightly (0.84-1.13  $\mu\text{M}$ ) depending upon the plasma volume (recommended range, 235-315 mL).

For MB photoinactivation of plasma to be centralized, but plasma from remote sites used as a start material, it is essential to be able to freeze and thaw plasma before treatment. Although we have previously evaluated the use of the two systems (Baxter and Maco Pharma) using fresh plasma,<sup>10-12</sup> we have not evaluated freeze-thawing of plasma before MB treatment using such systems. Although it is known that freeze-thawing itself has minimal effect on the coagulation factor activity of plasma,<sup>7</sup> there are no comparative data available on whether the loss of coagulation factor activity due to the MB inactivation step is affected by prior freeze-thawing. Furthermore, in the UK, there was concern that freeze-thawing non-WBC-reduced plasma could potentially increase exposure to vCJD due to fragmentation of platelets and WBCs, which are known to contain normal cellular prion protein (Prp<sup>c</sup>)<sup>13</sup> and might therefore host the infective abnormal prion protein Prp<sup>sc</sup>. We therefore assessed the effect of removing these cells by an additional WBC reduction step before freezing on the quality of MB plasma.

Following concerns over possible side effects of residual MB in plasma, a further recent development is the ability to remove MB by filtration before final component storage. Evaluations of two removal filters (Pall MB1, Pall Biomedical, Portsmouth, UK, and HemaSure LeukoVir, Marlborough, MA) MB have been previously

reported,<sup>14,15</sup> but there are no data available on the use of a new MB removal filter (Maco Pharma Blueflex). The aim of this study was therefore to evaluate the combined effect of WBC reduction before freezing, freeze-thawing, MB photoinactivation, and MB removal using two different filters, on coagulation factor activity and activation markers in FFP. We also examined the effect of freeze-thawing and subsequent filtration of non-WBC-reduced plasma on its cellular constituents, to provide assurance that the process is not likely to increase the risk of vCJD transmission after transfusion to patients.

## MATERIALS AND METHODS

### Blood collection and processing

The experimental design is shown in Fig. 1. Twenty-four units of whole blood (group A,  $n = 12$ ; group O,  $n = 12$ ) were collected into "Top and Bottom" configuration blood packs (Pall Medsep 789-94 U, Pall Biomedical). Blood was then centrifuged (Heraeus Cryofuge 6000, Kleinostheim, Germany) at 3300 rpm for 12 minutes at 22°C and processed to RBCs and plasma (Compomat G4 system, Fresenius-Hemocare NPBI, Abingdon, UK). In Experiment A, plasmas were pooled in groups of two units of identical ABO group into 600-mL transfer packs (Baxter FGR2089, Baxter Healthcare). The pools were mixed thoroughly and divided equally between two 300-mL transfer packs (Fresenius Hemocare P4164, Fresenius Hemocare). All units of plasma were frozen within 8 hours of collection in a freezer (Thermogenesis MP1101, Cheshire, UK) to  $-45^\circ\text{C}$  within 45 minutes and stored frozen at  $-40^\circ\text{C}$  for 4 days to 4 weeks. The units were then thawed at  $37^\circ\text{C}$  and immediately WBC reduced (PLAS 4, Maco Pharma, Middlesex, UK) and MB photoinactivated (Maco Pharma Maco-Tronic system) as previously described.<sup>11</sup> For each pair of plasmas, MB was removed (either Pall MB1 or Maco Pharma Blueflex) according to the manufacturers' instructions. Plasmas were refrozen in a freezer (Thermogenesis MP1101) and stored at  $-40^\circ\text{C}$ . In addition, 19 units of plasma were WBC reduced before freezing using one of two filters (RZ2000, Baxter Healthcare, or LPS1, Pall Biomedical), MB inactivated, and then MB removed (MB1 filter, Experiment B). These filters were selected because they are known to have minimal effect on coagulation factors in plasma.<sup>16</sup> A further 16 units of plasma (group O,  $n = 8$ ; group A,  $n = 8$ ) were MB inactivated without the freeze-thaw step and MB removed (Blueflex filter, Experiment C).

We took 15-mL samples by sterile connection of a sample pouch at four time points: 1) before freezing, 2) after thawing and before WBC reduction and MB addition, 3) after MB treatment before MB removal, and 4) after MB removal. Samples were frozen at  $-80^\circ\text{C}$  for coagulation assays, and two aliquots were frozen in EDTA for C3a des arg assays.

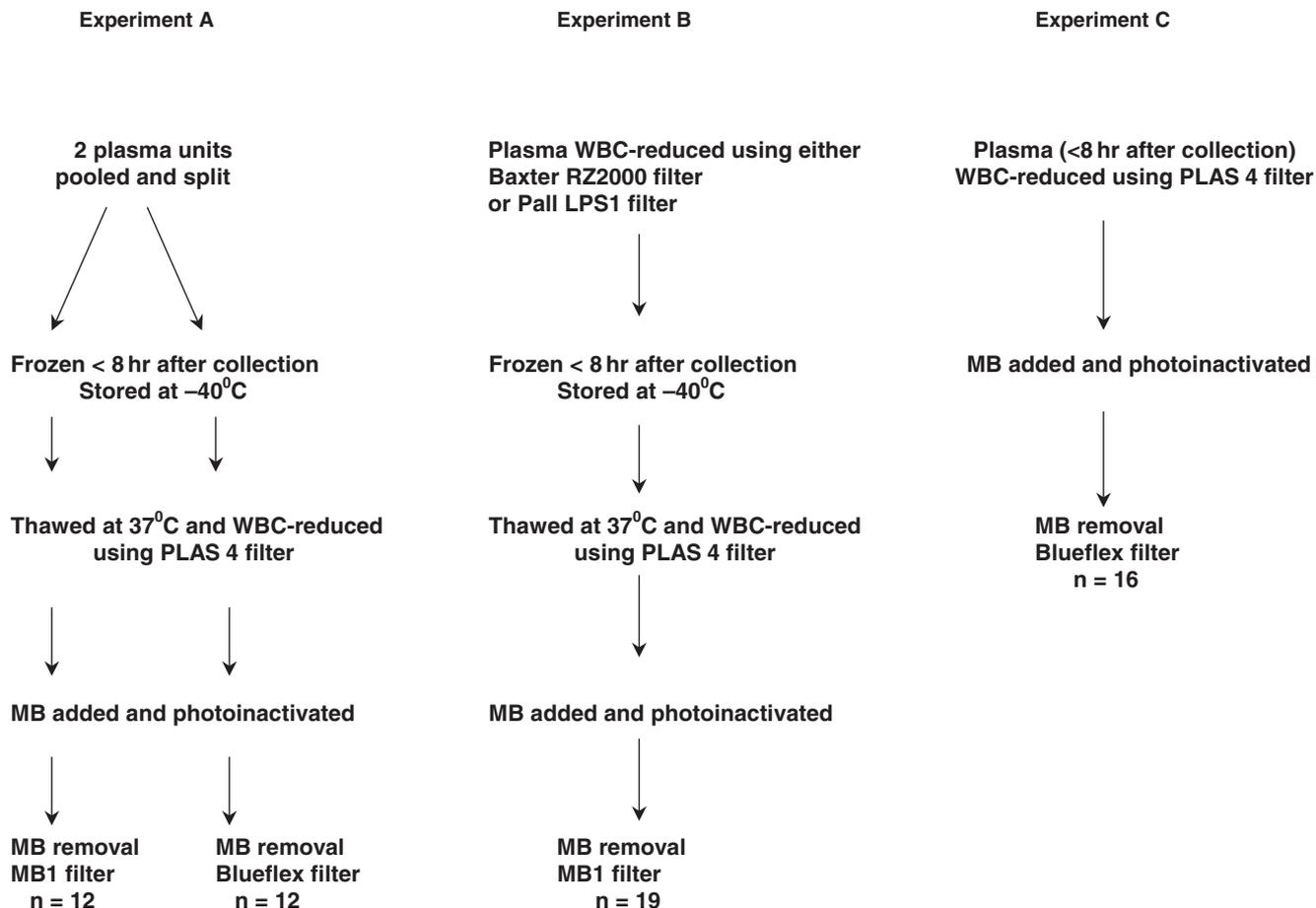


Fig. 1. Study design.

### Plasma factors

All coagulation assays were performed using commercially available analyzers (Sysmex CA 1500 analyzer, Sysmex, Milton Keynes, UK; Coagamate X2 analyzer, Organon-Teknika, Cambridge, UK; or Amelung KC 4 A micro analyzer, Sigma Diagnostics, Poole, Dorset, UK). FII, FV, FVII, and FX were assayed by one-stage prothrombin time (PT)-based assays and F IX and FXII using a one-stage activated partial thromboplastin time (APTT)-based assay, using deficient plasma (Dade Behring, Marburg, Germany). The PT and APTT were expressed as a ratio to the geometric mean result of 20 normal citrated plasmas. These types of samples were chosen as "normal" plasma to provide a standard reference point between studies. VWF antigen was measured by latex agglutination (STA Liatest Kit, Diagnostica Stago, Asnieres, France). FVIII and FXI were assayed using one-stage clotting assays with deficient plasma (Diagnostics Scotland, Edinburgh, Scotland; and Sigma-Aldrich Company, Poole, Dorset, UK, respectively). Fibrinogen was measured using a Clauss assay with Fibriquick reagents (Organon-Technika, Cambridge, UK). FVIII assays were standardized using the British

plasma standard (NIBSC, South Mimms, UK). All other assays were standardized using Coagulation Reference plasma 100 percent (Technoclone, Dorking, UK). A control plasma of known potency was assayed on each occasion for all coagulation assays.

Commercially available ELISA kits were used to determine levels of prothrombin fragment 1 + 2 (Dade-Behring), FXIIa (Axis-Shield, Dundee, Scotland), and VWF collagen-binding activity (VWF:CB, Immuno, Vienna, Austria). C3a des arg was assayed by radioimmunoassay (Amersham Pharmacia Biotech, Buckshire, UK). VWF cleaving protease activity was measured as previously described<sup>17</sup> and results expressed as a ratio to that of a pooled normal citrated plasma.

### Effect of freeze-thawing plasma and filtration steps on cellular content of plasma

Double WBC-reduced plasma (LPS1 filter, Pall Biomedical) was spiked with WBCs ( $<1-200 \times 10^6/L$ ) with or without platelets ( $<1-100 \times 10^9/L$ ), both of which were prepared from fresh whole blood by density gradient centrifugation,

to represent levels of cellular contamination that may be expected to occur in non-WBC-reduced plasma. Plasma was then blast-frozen, thawed, WBC-reduced by sterile connection with the (PLAS 4) filter, MB added, and MB removed using a filter (the Pall MB1 filter). Samples were taken at four stages: before freezing, after thawing, after PLAS4 LD filter, and after MB removal. Samples were analyzed for platelet count by a hematology analyzer (Sysmex SE9000, Sysmex) and WBC count by flow cytometry (FACSCalibur, Becton Dickinson, Oxford, UK) using LeucoCount reagents (Becton Dickinson). Release of the neutrophil primary granule marker elastase was measured by ELISA of  $\alpha_1$ -proteinase inhibitor: neutrophil (PMN) elastase complexes (Pathway Diagnostics, UK) and release of LDH by enzymatic assay in supernatant plasma (Vitros DT60II, Axis-Shield, Dundee, Scotland). RBC microparticles were measured by flow cytometry (FACSCalibur) as previously described using antibodies to glycophorin A.<sup>18</sup>

Analysis of platelet microparticles (PMP) was determined as follows: Plasma (5  $\mu$ L) was incubated for 20 minutes at room temperature with 5  $\mu$ L allophycocyanin-conjugated anti-CD61 (APC-CD61, Caltag-MedSystems, Towcester, UK), 5  $\mu$ L rhodophycocerythrin-conjugated anti-CD42b (Caltag-MedSystems), 10  $\mu$ L FITC annexin V (FITC-AV, Caltag-MedSystems), 5  $\mu$ L 10  $\times$  HBSS (Sigma, Poole, UK) and made up to 50  $\mu$ L with HEPES-calcium buffer (2.8 mM CaCl<sub>2</sub>, 20 mM HEPES). Samples were resuspended in 0.45 mL of 1  $\times$  HBSS and transferred to a tube containing a known amount of beads (TruCount, Becton Dickinson), and analyzed using a flow cytometer (FacsCalibur, Becton-Dickinson). Platelet microparticles were defined using forward scatter as events falling in a region, which includes less than 2 percent of platelets in plasma (PRP) from 20 normal donors, and of less than 1  $\mu$ m as determined by APC fluorescent beads (Spherotec, Libertyville, IL). Platelet-derived events were defined by fluorescence due to APC-CD61 binding above that of an isotype-matched control. Annexin-V-positive events were defined as events binding FITC-AV above a control containing 5 mM Na<sub>3</sub> EDTA. In normal subjects (n = 20), less than 1 percent of unstimulated platelets bind FITC-AV. To control assay variability, a negative control of unstimulated PRP and a positive control (PRP incubated with 10  $\mu$ M A23187 [Calbiochem-Novabiochem, San Diego, CA] for 15 min) were included for platelet microparticle assays.

### Effect of MB on assays

To assess the effect of the presence of MB itself on coagulation factor assays, a MB pellet (from the Maco Pharma pack) was dissolved in each of six units of plasma. Samples were collected before and after addition of MB, and once MB had been added, the plasma was not photoilluminated and was protected from light at all times. All parameters were performed as for the main study with the

exception that all coagulation assays were performed using a particular analyzer (Sysmex CA 1500 analyzer) and C3a des arg levels were performed by ELISA (Quidel, San Diego, CA).

### Statistical analysis

Since the distribution of some data were non-Gaussian with positive skew, nonparametric tests were applied. The Wilcoxon rank sum test was used for paired data and the Mann-Whitney U-test for unpaired data. A p value less than 0.05 was considered significant. All results are given as median with range.

## RESULTS

### Plasma processing

From the 24 paired units (Experiment A), one unit of plasma fractured on thawing, therefore data on 11 paired units of plasma are presented, all of which were within the required volume range before MB inactivation. Due to sampling, 2 out of 16 fresh plasma units (Experiment C) were slightly below the lower range limit (233 and 234 mL). Filtration time for one WBC-reduction filter (PLAS 4) was 8 to 15 minutes, with a loss of 20 mL of plasma. Filtration times for two other removal filters (Pall MB1 and Maco Blueflex) were 2 to 5 minutes and 5 to 11 minutes, respectively, with a loss of 10 mL of plasma for each.

### Effect of freeze-thawing and MB on loss of coagulation factors

The change in coagulation activity due to freeze-thawing and the MB process is shown in Table 1. There was a significant loss of FXII (2%) and VWF:CB (9%), and a small increase in levels of FVII (1%) and FXI (4%) due to freeze-thawing. This was associated with an increase in both PT ratio (1.06 [0.97-1.12] vs. 1.05 [0.95-1.11],  $p < 0.0001$ ) and APTT ratio (1.04 [0.91-1.20] vs. 1.02 [0.89-1.18],  $p < 0.0001$ ). The degree of loss of coagulation activity due to the MB process varied between factors, the highest losses occurring with FVIII (29%), fibrinogen (28%), and FXI (25%), therefore the evaluation of plasma treated fresh was mainly restricted to these factors. For FV, FVIII, FXI, and fibrinogen, the loss of activity due to the MB process was approximately 8-percent higher in the frozen-thawed plasma units compared with fresh plasma (Table 1). In addition, the increase due to this step in both PT ratio (0.09 [0.04-0.16] fresh vs. 0.14 [0.08-0.28] frozen-thawed,  $p < 0.0001$ ) and APTT ratio (0.11 [0.05-0.15] fresh vs. 0.16 [0.11-0.26] frozen-thawed,  $p < 0.0001$ ) was also higher. However, there was no significant difference in changes in levels of FVII, FXIIa, and C3a due to MB treatment between units which were treated fresh or after freeze-

**TABLE 1. Percentage change in coagulation factor activity due to freeze-thawing and MB treatment of plasma**

Factor	Due to freeze-thawing median (range)	Due to MB treatment + WBC reduction* (freeze-thawed plasma) median (range)	Due to MB treatment + WBC reduction (fresh plasma) median (range)
Number	22	22	16
Fibrinogen (g/L)	-10 (-27 to 14)	-28 (-51 to -20)	-21 (-38 to -7)†
FII (IU/mL)	0 (-4 to 4)	-8 (-11 to -2)	NA
FV (U/mL)	0 (-5 to 4)	-13 (-20 to 4)	-5 (-11 to 4)†
FVII (IU/mL)	1 (-1 to 4)‖	-7 (-10 to -1)	-4 (-9 to -1)
FVIII (IU/mL)	-3 (-20 to 9)	-29 (-42 to -9)	-24 (-37 to -11)†
F IX (IU/mL)	1 (-3 to 4)§	-13 (-20 to -11)	NA
FX (IU/mL)	0 (-2 to 3)	-15 (-22 to -10)	NA
FXI (U/mL)	4 (-19 to 11)‖	-25 (-35 to -7)	-15 (-23 to -6)†
FXII (U/mL)	-2 (-6 to 1)‖§	-18 (-31 to -14)	NA
VWF:Ag (IU/mL)	-1 (-4 to 3)§	-6 (-11 to -3)	NA
VWF:CB (U/mL)	-9 (-17 to 5)‖§	-8 (-16 to 3)	NA
FXIIa (ng/mL)	0 (-25 to 43)	-20 (-43 to 33)	-14 (-43 to 0)
Prothrombin <i>F1 + 2</i> (nM)	-19 (-55 to 32)¶	91 (36 to 160)	27 (-12 to 180)‡
C3a des arg (ng/mL)	0 (-41 to 65)	-10 (-45 to 155)	16 (-27 to 295)

\* WBC reduction was performed with an integral PLAS 4 WBC-reduction filter in the Maco Pharma MB pack configuration.

†  $p < 0.01$  refers to significance from the Mann-Whitney U-test between fresh and frozen plasma.

‡  $p < 0.05$ .

§  $n = 11$ .

¶  $p < 0.01$  refers to significance from the Wilcoxon rank sum test between plasma before freezing and after thawing.

‖  $p < 0.05$ .

thaw. The increase in prothrombin *F1 + 2* levels due to MB treatment was higher in frozen-thawed units compared to fresh.

When the influence of MB on the assays was studied (in the absence of photoinactivation), there was no significant difference between before or after the addition of MB for any parameters, apart from FXIIa, which was significantly lower after MB addition (1.59 [0.76-2.13] ng/mL before, 0.72 [0.56-1.05] ng/mL after,  $p < 0.05$  before vs. after).

### Effect of MB removal filters on coagulation activity

To assess the difference between the two MB removal filters, pairs of units were pooled and half of each pool MB-treated and processed through each of the removal filters in parallel. Due to logistical problems, it was not possible to process and assay these two sets simultaneously. A small difference was apparent in levels of some coagulation factors between the two arms of the study, probably due to small differences in processing and storage. However, the percentage change in activity due to freeze-thawing and MB treatment was the same for each arm of the study (data not shown), and therefore these data were combined. To evaluate the effect of MB removal filters, a comparison of pre- and postcoagulation activity for each arm of the study was examined (Table 2). There was no apparent decrease in any parameter studied with either removal filter, apart from a reduction in levels of C3a using one of the filters (Pall MB1). There was a slight increase in levels of fibrinogen, FII, FV, FVII, and FX, using the Pall filter. There was an apparent increase in levels of FXIIa

after filtration with both removal filters, which was probably due to the influence of MB on the assay and was comparable between filters. There was an extremely small variation in the PT and APTT ratios with both filters (Table 2).

We compared the final levels of coagulation activity in frozen-thawed MB-treated plasma to a reference range based on 66 samples of WBC-reduced plasma that had not been MB treated. Because there was no loss of activity with either MB removal filter, both sets of data were pooled. The reference data was not collected as part of this study but from previous studies carried out by the National Blood Service over the past 4 years. The methodology used in these studies<sup>16</sup> for either plasma processing or assay did not differ significantly from the current study. Over 90 percent of MB units were within our reference range for all coagulation factors, apart from prothrombin *F1 + 2* and PT ratio, the MB-treated plasma having 23 percent and 50 percent of values above the range, respectively (Table 3). The range of PT ratios observed in reference and MB-treated plasma is shown in Fig. 2.

We did not evaluate MB removal by the filters used in this study, but previous studies have shown that the MB1 filter removes 81 to 95 percent of MB<sup>14</sup> and the Blueflex filter removes more than 95 percent MB.<sup>19</sup>

### Effect of WBC reduction before freezing

WBC reduction of plasma before freezing appeared to have little influence on final levels of FVIII (0.67 [0.44-1.23] WBC reduced vs. 0.62 [0.48-0.86 IU/mL] non-WBC reduced) or fibrinogen (1.88 [1.45-3.24] WBC reduced vs.

**TABLE 2. Effect of MB-removal filters on plasma coagulation activity using frozen-thawed MB photoinactivated plasma**

Factor	MB1 filter median (range)		Blueflex filter median (range)	
	Before MB removal	After MB removal	Before MB removal	After MB removal
Number		11		11
PT (ratio)	1.17 (1.11-1.34)	1.07 (1.03-1.24)*	1.23 (1.13-1.33)	1.22 (1.13-1.32)*
APTT (ratio)	1.11 (1.05-1.28)	1.13 (1.06-1.30)†	1.25 (1.17-1.39)	1.27 (1.19-1.43)*
Fibrinogen (g/L)	1.88 (1.30-2.13)	1.93 (1.28-2.27)*	2.04 (1.37-2.13)	1.96 (1.28-2.32)
FII (IU/mL)	0.96 (0.77-1.04)	1.00 (0.78-1.04)†	0.95 (0.77-1.04)	0.97 (0.77-1.04)
FV (U/mL)	0.78 (0.55-0.86)	0.80 (0.56-0.91)*	0.76 (0.58-0.97)	0.76 (0.58-0.88)
FVII (IU/mL)	0.99 (0.79-1.43)	1.02 (0.83-1.55)*	1.01 (0.77-1.40)	1.00 (0.78-1.45)
FVIII (IU/mL)	0.63 (0.47-0.89)	0.62 (0.48-0.86)	0.61 (0.48-0.79)	0.61 (0.46-0.76)
F IX (IU/mL)	0.96 (0.78-1.06)	0.96 (0.83-1.11)		
FX (IU/mL)	0.95 (0.70-1.12)	1.02 (0.75-1.15)*	0.95 (0.72-1.09)	0.96 (0.73-1.07)
FXI (U/mL)	0.77 (0.57-0.99)	0.77 (0.59-0.99)	0.75 (0.55-0.99)	0.71 (0.58-0.78)
FXII (U/mL)	0.88 (0.41-1.07)	0.88 (0.40-1.09)		
VWF:Ag (IU/mL)	0.99 (0.74-1.19)	0.98 (0.75-1.18)	0.92 (0.70-1.09)	0.93 (0.70-1.09)
VWF:CB (U/mL)	0.60 (0.47-0.68)	0.73 (0.42-0.85)	0.70 (0.59-0.87)	0.74 (0.56-0.82)
FXIIa (ng/mL)	1.50 (0.40-2.50)	1.80 (0.70-2.90)*	1.25 (0.75-2.00)	1.50 (1.00-2.25)*
Prothrombin F1 + 2 (nM)	0.96 (0.61-1.75)	0.88 (0.76-1.83)	0.74 (0.58-1.19)	0.75 (0.52-1.25)
C3a des arg (ng/mL)	438 (207-1928)	304 (107-431)*	337 (225-1909)	302 (226-1713)

\* p < 0.01 refers to statistical significance from the Wilcoxon rank sum test between plasma before and after MB removal.  
 † p < 0.05.

**TABLE 3. Final levels of coagulation factors and activation markers in freeze-thawed MB-photoinactivated plasma after MB removal**

Factor	Final level in plasma*	Reference range†	Units in range (%)
Number	22	66	
PT (ratio)	1.16 (1.03-1.32)	1.05 (0.95-1.16)‡	50
APTT (ratio)	1.24 (1.06-1.43)	1.09 (0.86-1.36)‡	95
Fibrinogen (g/L)	1.95 (1.28-2.32)	1.10-4.30	100
FII (IU/mL)	0.98 (0.77-1.04)	0.70-1.20	100
FV (U/mL)	0.79 (0.56-0.91)	0.50-1.40	100
FVII (IU/mL)	1.01 (0.78-1.55)	0.60-1.40	100
FVIII (IU/mL)	0.62 (0.46-0.86)	0.40-1.60	100
F IX (IU/mL)	0.96 (0.83-1.11)§	0.60-1.40	100
FX (IU/mL)	1.01 (0.73-1.15)	0.70-1.30	100
FXI (U/mL)	0.75 (0.58-0.99)	0.60-1.30	91
FXII (U/mL)	0.88 (0.40-1.09)§	0.40-1.50	100
VWF:Ag (IU/mL)	0.96 (0.70-1.18)	0.60-1.65	100
VWF:CB (U/mL)	0.74 (0.42-0.85)	0.50-1.50	91
FXIIa (ng/mL)	1.50 (0.40-2.90)	0.50-5.00	100
Prothrombin F1 + 2 (nM)	0.85 (0.52-1.83)	0.20-1.10	77
C3a des arg (ng/mL)	303 (107-1713)	1117 (0-12,330)	100

\* Data (n = 22) are represented by the median (range) from plasma MB-removed by MB1 filter (n = 11) and Blueflex filter (n = 11).  
 † Reference range of normal plasmas is defined as the mean ± 2 SD for normally distributed data and the geometric mean with 95-percent CI for skewed data based on WBC-reduced FFP. Percentage of units in range is defined as above the lower limit for coagulation factors and below the upper limit for PT, APTT, and activation markers.  
 ‡ n = 100.  
 § n = 11, using MB1 filter only.

1.70 [1.24-2.31 g/L] non-WBC reduced) in plasma subsequently MB treated and removed using the MB1 filter. VWF cleaving protease (VWF:CP) activity was measured in four MB-inactivated plasmas, which were WBC reduced before freezing and MB-removed using the MB1 removal filter. VWF:CP results ranged from 0.81 to 1.00 (normal range, 0.80-1.20 in citrated plasma). We have not assessed VWF:CP activity in plasma MB-depleted using the Blueflex filter. Quality-monitoring data from routinely processed

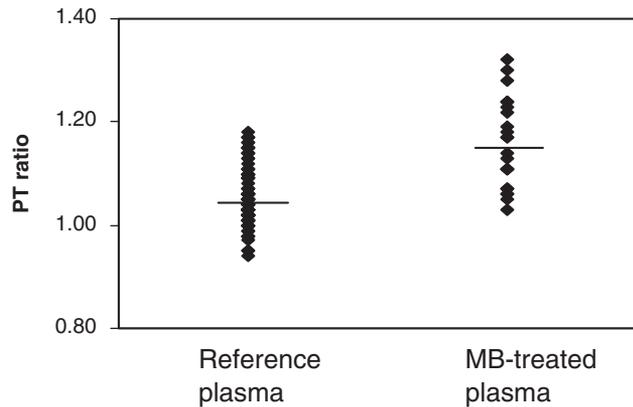
units (n = 225), WBC-reduced before freezing, MB-treated, and MB-removed using the MB1 filters, showed a mean volume of 242 mL (SD = 19) and FVIII content of 0.79 IU per mL (SD = 0.25). These results comply with UK specifications for MB-treated FFP<sup>20</sup>

**Effect on cellular content of plasma**

When platelets were spiked into plasma, there was no consistent difference between levels measured before and after freeze-thawing when measured by hematology analyzer, but levels were consistently lower after thawing when measured by flow cytometry (Table 4). When WBCs were spiked into plasma, there appeared to be a trend for lower WBC counts and higher levels of α<sub>1</sub>-proteinase inhibitor: PMN elastase complexes in plasma after freezing (Table 4). However, in the absence of platelets, levels of LDH did not increase substantially. Freeze-thawing of plasma

resulted in an increase in levels of platelet microparticles as well as microparticles characterized by the binding of purified annexin V (Table 4). Platelet microparticles were reduced to levels observed in fresh WBC-reduced plasma or below after WBC reduction of frozen plasma with the PLAS 4 filter. However, a significant proportion (~30%) of microparticles characterized by annexin V binding were not removed by the WBC-reduction or the MB1 removal step. These also appeared to be derived solely from plate-

lets because levels after WBC reduction in samples spiked with WBCs in the absence of platelets were not different from WBC-reduced plasma alone. However, even at an added platelet count of  $30 \times 10^9$  per L (Sample D, the cur-



**Fig. 2.** PT ratio in MB-treated (n = 22) or reference plasma (n = 100). For MB-treated plasma, MB was removed by MB1 filter (n = 11) and Blueflex filter (n = 11). Reference plasma is historical data from WBC-reduced FFP. Horizontal bar represents the median value. The PT is expressed as a ratio to the geometric mean result of 20 normal citrated plasmas.

rent UK specification), the number of annexin V-positive microparticles in frozen-thawed plasma subsequently filtered using the PLAS 4 filter ( $43 \times 10^9/L$ ) is similar to that seen in our current routine WBC-reduced non-MB-treated plasma product (mean residual platelet count of  $3 \times 10^9/L$ ).

Before freezing, levels of RBC microparticles were 8 ( $5-12 \times 10^9/L$ ), increasing by 33 percent after freeze-thawing. This was not related to platelet or WBC content, and levels after WBC reduction were below the detection of the assay system used (data not shown).

### DISCUSSION

MB treatment of plasma has been shown to inactivate 4 to 6 logs of transfusion-transmitted viruses, including HIV, HBV, parvovirus B19, and West Nile virus.<sup>5,21,22</sup> Original studies on MB inactivation were reported on plasma freeze-thawed before treatment.<sup>5,7</sup> Later, work on other systems (Baxter Pathinact and Maco Pharma Theraflex systems) was performed on fresh plasma.<sup>10-12</sup> However, there are no data available on the difference between using fresh or freeze-thawed plasma as a starting component for MB treatment. In our study, freeze-thawing of plasma resulted in a small loss of FXII and VWF:CB activity

**TABLE 4.** The effect of freeze-thawing, WBC reduction, and MB removal on cellular components of plasma

	Spike*									
	A	B	C	D	E	F	G	H	I	J
Platelets – HA† ( $10^9/L$ )										
Before freeze	<3	<3	10	31	108	<3	<3	<3	<3	<3
After thaw	<3	6	10	32	97	<3	<3	<3	<3	<3
Platelets – FC‡ ( $10^9/L$ )										
Before freeze	<0.5	5.9	10.8	32.0	125.1	<0.5	<0.5	<0.5	<0.5	<0.5
After thaw	<0.5	4.8	9.0	24.6	92.2	<0.5	<0.5	<0.5	<0.5	<0.5
WBCs ( $10^9/L$ )										
Before freeze	<1	8.9	29.6	90.7	233.8	<1	1.8	14.1	50.6	92.9
After thaw	<1	9.1	28.1	94.0	210.9	<1	1.6	13.2	43.4	89.5
PMN elastase ( $\mu g/L$ )										
Before freeze	46.4	39.7	37.5	46.3	57.0	20.6	23.7	24.0	25.3	25.7
After thaw	37.5	35.8	42.2	53.5	79.8	22.8	23.1	30.4	43.2	58.8
After WBC reduction	34.5	35.3	40.8	57.4	77.4	21.1	20.5	27.0	41.2	53.5
After MB1 filter	35.1	37.0	45.1	58.2	71.9	18.1	21.3	24.8	37.9	57.9
Platelet microparticles ( $10^9/L$ )										
Before freeze	0.2	0.2	0.4	0.5	1.0	0.3	0.5	0.4	0.3	0.9
After thaw	0.1	0.5	0.7	2.6	10.1	0.1	0.1	0.0	0.3	0.0
After WBC reduction	0.0	0	0	0.1	0.2	0	0	0	0.1	0
After MB1 filter	0.1	0.0	0.1	0.1	0.2	0.1	0.0	0.1	0.0	0.3
Annexin V +ve microparticles ( $10^9/L$ )										
Before freeze	6	7	8	10	13	4	4	4	5	4
After thaw	12	31	54	138	574	9	9	9	13	13
After WBC reduction	3	10	20	46	198	5	2	3	5	5
After MB1 filter	3	8	15	43	196	4	4	4	4	8
LDH (U/mL)										
Before freeze	393	400	423	476	732	390	381	388	376	382
After thaw	384	404	434	523	796	388	386	385	393	395

\* WBC-reduced plasma units were spiked with WBCs alone (Samples G-J) or WBCs and platelets (Samples B-E) to the concentrations shown in the before freeze rows. Samples A and F were not spiked. Plasma was frozen-thawed, WBC reduced using the PLAS 4 filter; MB added and MB removed using the MB1 filter. Results are from a single experiment. Platelets and WBCs were not detectable following WBC reduction.

† FC-flow cytometry.

‡ HA-haematology analyser.

as well as a minor prolongation of PT and APTT. Interestingly, there was an apparent increase in activity of FVII and FXI on freeze-thawing, presumably reflecting small changes in the activation status of these factors. However, other factors studied remained unchanged. This agrees with the work of Zeiler et al.,<sup>7</sup> who showed that the loss of coagulation activity in MB-treated plasma was mainly attributable to the MB photoinactivation step rather than freeze-thawing of plasma. The loss of activity observed in frozen-thawed units due to MB photoinactivation in our study was similar to that previously reported.<sup>5,7</sup> For the variables we studied, with the exception of FVII, loss of coagulation factor activity due to the MB-inactivation step (including the WBC reduction filter) was 8-percent higher when frozen-thawed plasma units were used rather than fresh. In addition, the increase in prothrombin *F1 + 2* levels after MB inactivation and WBC reduction was higher in units frozen-thawed compared with fresh plasma, indicating a higher degree of thrombin generation. This was not associated with an increase in FXIIa. Because our MB process includes an integral WBC-reduction step, we cannot determine whether the differences seen between fresh and frozen-thawed plasma are attributable to the WBC-reduction or MB process. The WBC-reduction filter used in the MB packs has previously been shown to have minimal effect on coagulation activity (unpublished data) using fresh plasma, but this could be different for frozen-thawed plasma.

We also sought to compare the effect of two different types of MB removal filters (Maco Pharma Blueflex or Pall MB1 filter) on plasma factor activity. Neither filter resulted in loss of any variable studied. Both MB removal filters resulted in a small increase in the APTT ratio, which might be attributable to contact activation of plasma with the filter. This is difficult to assess because although both filters increased FXIIa antigen, this assay is influenced by MB. However, levels of FXIIa antigen in the final MB-removed component were not higher than untreated plasma units. The filtration times for one filter (Maco Pharma Blueflex) were longer compared with the other (Pall MB1) (5-11 vs. 2-5 min, respectively), but the loss of plasma was equivalent for both filters. However, there did not appear to be any difference between the two filters in terms of activation of the contact system or thrombin generation as evidenced by the generation of FXIIa or prothrombin *F1 + 2*. Our results using the MB1 filter compare well with that previously published,<sup>14</sup> showing minimal loss of clotting factor activity. However, there was an apparent increase in levels of fibrinogen and PT-derived coagulation factors after filtration with the MB1 filter, which was associated with a small decrease in the PT ratio. We cannot explain these results because these assays do not appear to be influenced by the presence of MB, but they could possibly be a result of small increases in the activation state of coagulation factors. The changes in

coagulation factors observed after filtration with either MB-removal filter appear to be clinically insignificant. Neither MB-removal filter resulted in generation of C3a des arg, a marker of complement activation. However, levels after filtration were reduced using the Pall MB1 filter. Whether this has any clinical benefit in terms of acute reactions is unclear.

As well as examining the loss of coagulation factors during MB photoinactivation, we compared residual levels in the final component with reference ranges based on previous studies of nontreated FFP in our laboratories. Despite the observed losses of coagulation factors due to MB treatment, final levels of all coagulation factors in frozen-thawed, MB-treated, and removed FFP were above the lower limit of the reference range in over 90 percent of units. However, over 50 percent of units were above the upper reference range for PT ratio. This presumably reflects the loss of fibrinogen and FII, FV, FVII, and FX because the PT is dependent upon these factors. However, the PT ratio of all units was less than 1.35. In addition, 23 percent of units had levels of prothrombin *F1 + 2* higher than the upper limit of the reference range, reflecting the increase in prothrombin *F1 + 2* seen due to the MB process. The clinical significance of increased *F1 + 2* levels is unclear, but values higher than those observed in our study are seen in S/D-treated plasma.<sup>23</sup> We did not assess the effect of MB treatment or removal on plasma inhibitors of coagulation. However, MB treatment is reported to have minimal effect on levels of antithrombin,  $\alpha_2$ -antiplasmin and protein C & S.<sup>5,7,11</sup> After MB removal (Pall MB1 filter), levels of antithrombin and protein C & S are within the normal range.<sup>14</sup>

We also evaluated the addition of a WBC-reduction step before freezing plasma, which did not appear to augment the loss of fibrinogen and FVIII activity. VWF:CP activity in plasma that was WBC reduced, MB inactivated, and removed using the MB1 filter was also within reference ranges established by other laboratories, suggesting that these processing steps do not have a major effect on VWF:CP using the techniques employed. These results are consistent with our previous findings on MB-treated fresh plasma<sup>24</sup> and others results on frozen-thawed plasma.<sup>25</sup> However, we cannot exclude small losses of activity given the relatively small number of samples used in this study.

In the UK, all blood components are WBC-reduced before storage. However, for logistical reasons we also wanted the flexibility to freeze non-WBC-reduced plasma intended for MB treatment, as a subsequent WBC-reduction step is integral to the Maco Pharma MB process. We were concerned that freeze-thawing may result in reduced cell removal by the WBC-reduction filter or cause fragmentation of cells with the potential for increasing the risk of transmission of vCJD. Most cellular prion protein in blood, used here as a surrogate marker for the potentially infective abnormal prion protein, is associated with

plasma (65%), with 26.5 percent, 1.8 percent, and 0.8 percent associated with platelets, RBCs, and polymorphonuclear cells, respectively.<sup>13</sup> Our results indicate that the majority of WBCs can be detected after thawing, and these are removed to undetectable levels after the WBC-reduction step of the MB process. However, the method we employed to detect WBCs predominantly measures WBC nuclei (unpublished data), and therefore provides little information on cellular integrity. The majority of WBCs in freeze-thawed plasma are detectable with PI without prior permeabilization,<sup>26,27</sup> suggesting that freeze-thawing alters WBC membrane integrity. The increase in levels of  $\alpha_1$ -proteinase inhibitor: PMN elastase complexes after thawing of plasma spiked with WBCs shows that PMN degranulation is occurring, but the postthaw levels remain below 100  $\mu\text{g}$  per L, which is not suggestive of large-scale PMN disintegration. Furthermore, in the absence of platelets, levels of LDH did not increase substantially after freezing, suggesting that WBCs do not disintegrate. We were unable to assess WBC fragments due to the insufficient sensitivity of available methods.

When platelets were spiked into plasma, there was an increase in platelet-derived microparticles after freeze-thawing of plasma, which probably explains the small decrease in platelet count detected by flow cytometry because these events would not be included in the platelet count. This fall was not detected by hematology analyzer, possibly because cell fragments can be detected as platelets by impedance-based methods. These fragments were reduced to or below the level in WBC-reduced fresh plasma after the WBC-reduction step of the MB process. However, we also analyzed cell microparticles based on the binding of purified annexin V, which has a high affinity for anionic phospholipids. Freeze-thawing resulted in an increase in annexin-V-positive microparticles, which appear to be mainly derived from platelets and were only partially removed by WBC reduction. The increased detection of microparticles by this method compared with using an antibody against the platelet receptor CD61 is probably attributable to the greater number of molecules per platelet of anionic phospholipid ( $1 \times 10^6$ ) compared with CD61 ( $4\text{--}8 \times 10^4$ ).<sup>28,29</sup> The presence of RBC and WBC microparticles (which will also bind annexin V) may also help to explain this difference, but this seems unlikely because in the absence of platelets the differences between methods were less pronounced. The number of annexin-V-positive microparticles found in non-WBC-reduced plasma that has been frozen-thawed and then filtered is not appreciably higher than would be found in plasma that we currently produce.

The effect of loss of coagulation factor activity due to MB treatment on the *in vivo* efficacy of the component is difficult to assess because there are no published randomized, controlled clinical trial data comparing MB to either standard FFP or S/D-treated FFP. However, 2.5 million

units of MB FFP have been transfused internationally without obvious clinical sequelae.<sup>30,31</sup> In Spain, the switch from standard to MB-treated FFP has been associated with an increase in demand for FFP and cryoprecipitate,<sup>32</sup> which the authors attribute to loss of coagulation activity. However the increase in use (56%) appears to be disproportionate to the decrease in coagulation factors, suggesting that other factors, such as perception of a safer component, may have been influencing usage. It is also reported that the use of MB FFP is associated with a higher number of plasma exchanges compared with untreated FFP for the treatment of thrombotic thrombocytopenic purpura,<sup>33</sup> although we found no difference in the levels of VWF cleaving activity, the presumed therapeutic moiety in plasma treatment of thrombotic thrombocytopenic purpura, in MB FFP.<sup>24</sup> It is critical that transfusion services introducing pathogen inactivation of components monitor ongoing trends in usage as well as having a system for hazard reporting. At the time of writing, MB-treated and -removed FFP is routinely produced in England and Wales for transfusion to children and neonates born after 1995, with similar arrangements in other parts of the UK. However, in the near future, plasma to be pathogen inactivated for this patient group throughout the UK will be imported from volunteer donors in North America. Processes currently available for the pathogen inactivation of plasma all result in a decrease in coagulation factor activity. Improvements in the safety of blood need to be balanced against some likely reduction in the component potency. Single-unit systems for pathogen inactivation of plasma that have less effect on coagulation factor activity are clearly desirable.

#### ACKNOWLEDGMENTS

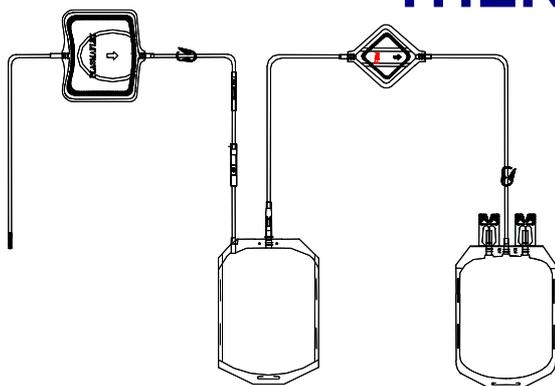
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# Pathogen Reduction System THERAFLEX - MB PLASMA



Pathogen Reduction of  
Leucodepleted Plasma  
Methylene Blue  
Removal by Filtration

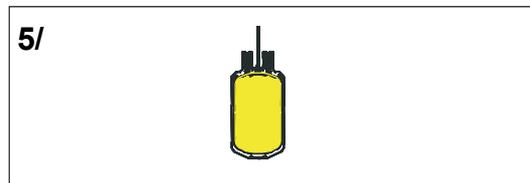
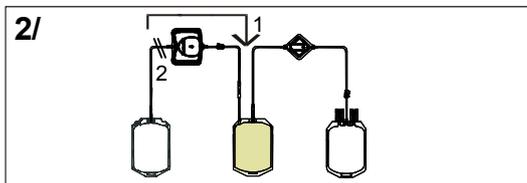
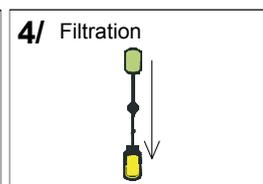
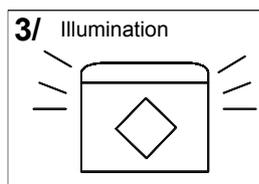
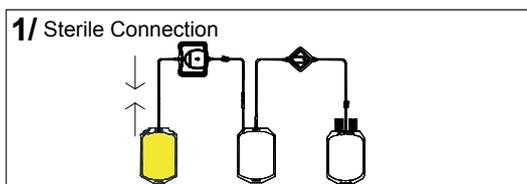


Ref. SDV0001XQ

## Specifications

- . **Filters** : Plasmafex PLAS4, Blueflex filter
- . **Bags** : 2 PVC
- . **Included Items** : Methylene Blue pill (85µg)
- . **Label** : English, French, German, Dutch
- . **Sterilisation** : Steam
- . **Shelf life** : 2 years
- . **Packaging** : 2 packs/peelable sachet  
- 24 packs/box

## Use



■ Whole Blood   
 ■ RCC   
 ■ PLASMA   
 ■ MB PLASMA   
 ■ PRP   
 ■ Platelets   
 ■ SAG-M PAGGSM   
 ■ NaCl   
 ■ Buffy Coat   
 ■ Serum   
 ■ Supernatant   
 ■ Cryoprecipitate