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

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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 (Maocotronic) 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
1. Gobal tests						
Quick	80 -130	%	98 ± 2	105 ± 3	94 ± 2	93 ± 3
INR			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
2. Coagulation factors						
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	050 - 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	%	97 ± 10	101 ± 11	99 ± 10	98 ± 11
F XIII	70 - 130	%	81 ± 11	77 ± 7	75 ± 4	85 ± 9
vWF (Ristoc. Co-Fr.)	50 - 140	%	85 ± 8	81 ± 19	94 ± 13	95 ± 7
3. Inhibitors						
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 - 150	%	110 ± 7	109 ± 6	116 ± 6	110 ± 3
Protein S	70 - 140	%	75 ± 7	70 ± 2	76 ± 2	81 ± 2
4. Fibrinolysis						
Plasmin inhibitor	80 - 120	%	103 ± 4	101 ± 3	103 ± 6	105 ± 10
alpha-1-Antirypsin	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
5. Complement						
CH50	70 - 100	%	116 ± 14	110 ± 11	108 ± 5	114 ± 8
C3a	123-2228	ng / ml	1029 ± 323	1106 ± 596	898 ± 332	921 ± 217
6. Activation						
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

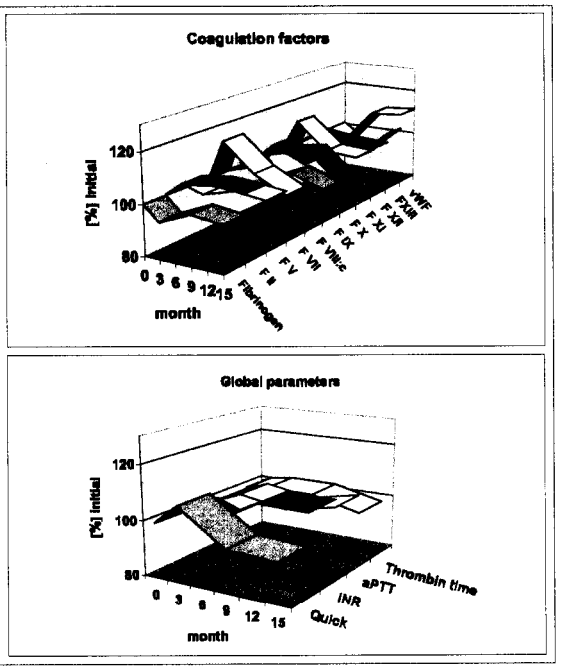

Fig. 1: Storage stability of Theraflex MB-Plasma

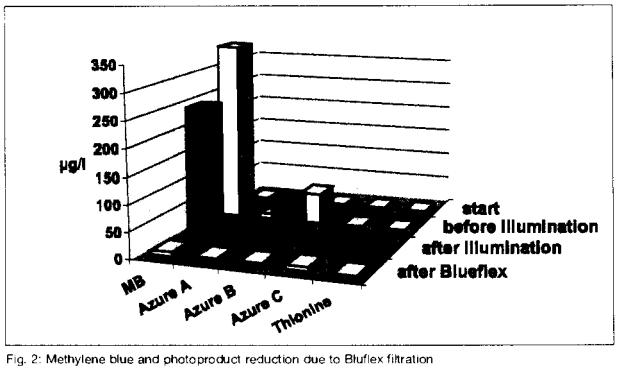

Fig. 2: Methylene blue and photoproduct reduction due to Bluflex filtration

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.

QUALITY OF THERAFLEX® MB-PLASMA DURING STORAGE AND TREATMENT

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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

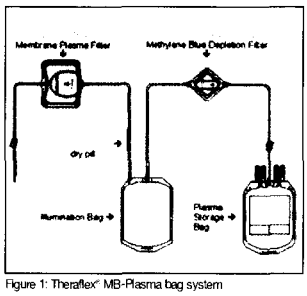

Figure 1: Theraflex® MB-Plasma bag system

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].

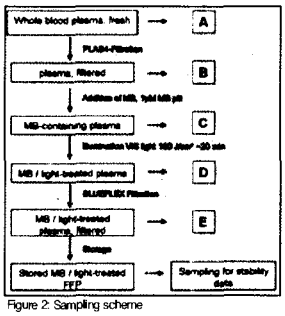

Figure 2: Sampling scheme

Results

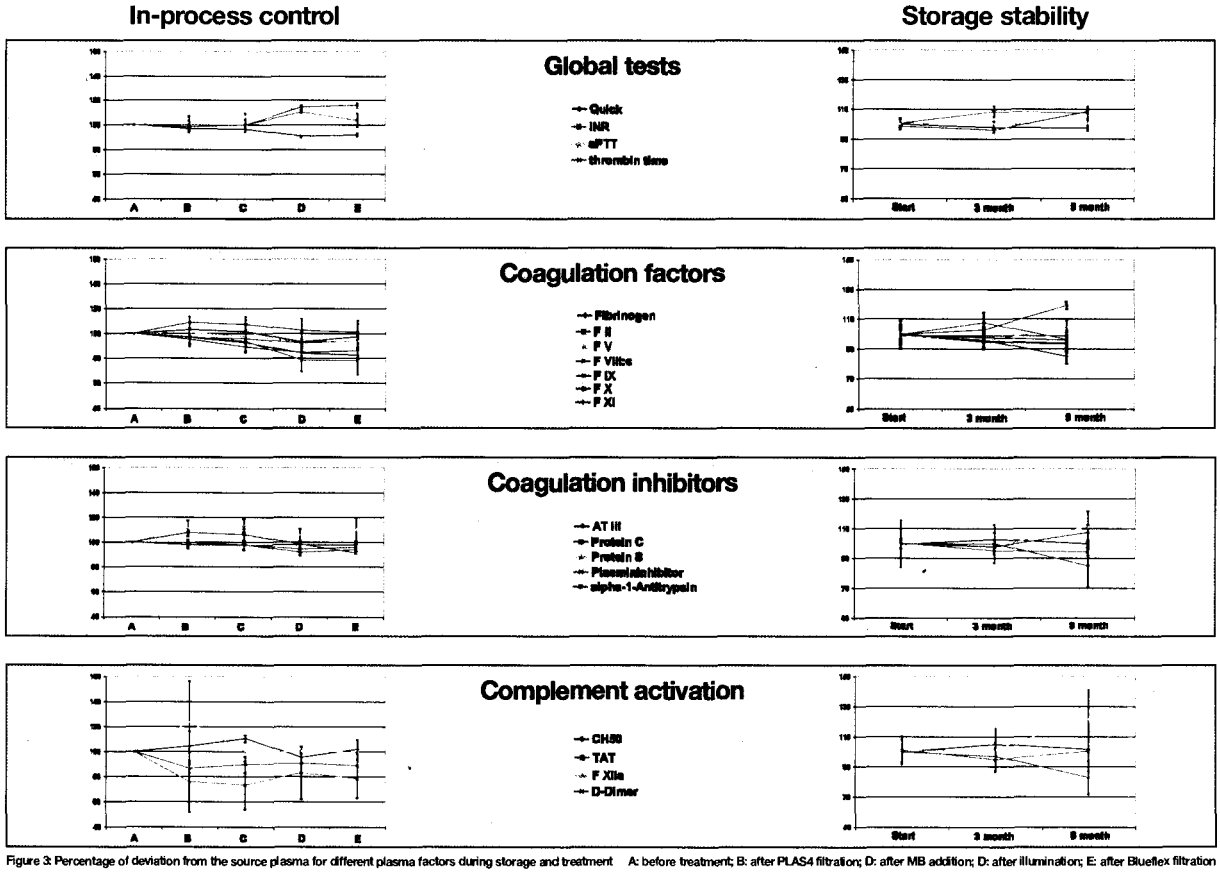

Figure 3: Percentage of deviation from the source plasma for different plasma factors during storage and treatment A: before treatment; B: after PLAS4 filtration; D: after MB addition; D: after illumination; E: after Blueflex filtration

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® 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® 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 (Clauss), 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 α_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 (Clauss)	[mg/dl]	229.0	37.6	222	41.9	-20.3
Factor V	[%]	120.7	9.0	101.8	20.8	-16.1
Factor VIII	[%]	147.2	23.0	89.3	21.3	-22.2
Factor XI	[%]	94.6	2.1	82.1	18.7	-13.3
Antithrombin III	[%]	87.6	2.1	87.3	6.2	-0.3
Protein C	[%]	105.3	24.3	94.5	17.1	-9.8
Protein S, free	[%]	94.2	11.2	94	12	-0.2
vWF:RCo	[%]	98.8	7.1	98.8	7.5	+0.0 *
vWF CP	[%]	87.8	13.1	90	11	+2.8
Plasmin inhibitor	[%]	94.5	9.1	95	8	+0.5
α_1-Antitrypsin	[mg/dl]	95.8	14.3	95.5	16	-0.3
F 1+2	[nmol/l]	1.07	0.75	1.06	0	-1.0 *
D-Dimers	[mg/l]	0.70	0.61	0.65	0.61	-7.5 *
CH 100	[U/ml]	0.68	1.15	1.33	1.09	+1.1

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

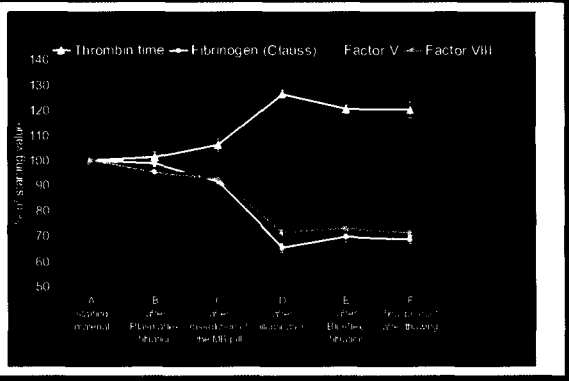


Fig.2 Influence of the individual manufacturing steps on plasma quality (data from three pools)

Methylene Blue	µmol/l	Depletion (C / A)
A (before illumination)	0.97 +/- 0.06	
B (after illumination)	0.63 +/- 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**	
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)

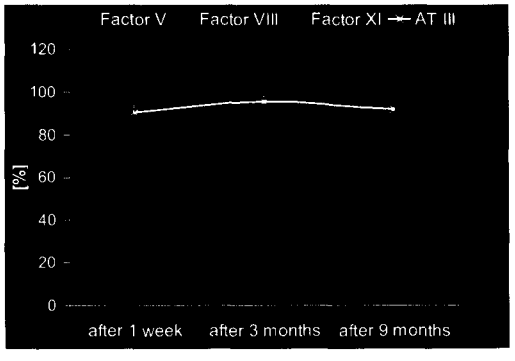
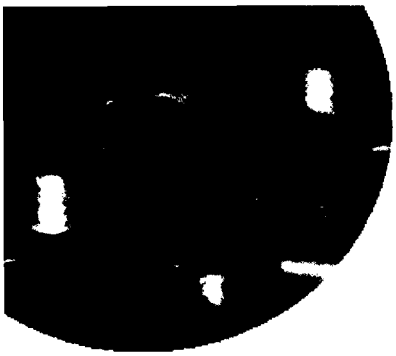


Fig.4 Stability of MB/light-treated plasma (data from 4 pools, , treatment under worst-case conditions)

CONCLUSIONS

Even under worst-case conditions, photodynamic treatment of FFP using the Theraflex MB-Plasma® 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

						For each stage in the preparation		
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
α2-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 (µg/l)	0.9 - 15.4	10.9	14.1	15.1	23.9	7.5	8.3	10.9
Factor XIIa (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

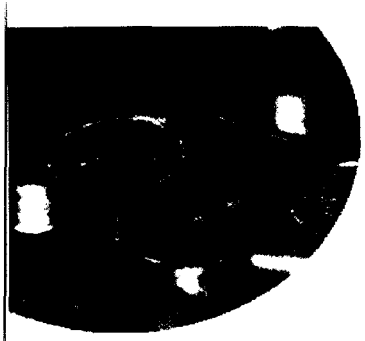
Ab : Plasma after visible light (plasmas 31 to 40)

B : MB-removed Plasma before freezing

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

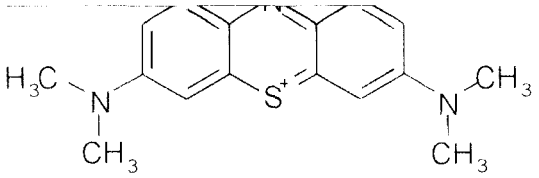
D : MB-removed Plasma after 6 months at - 30° C

MacoPharma

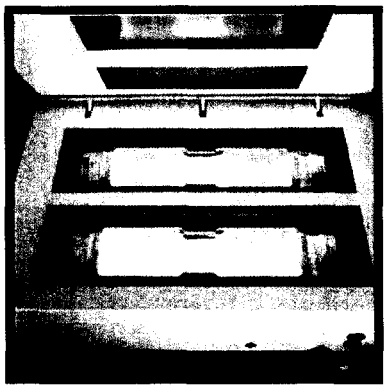


THERAFLEX-MB Plasma

Processing principle



Methylene Blue molecule



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

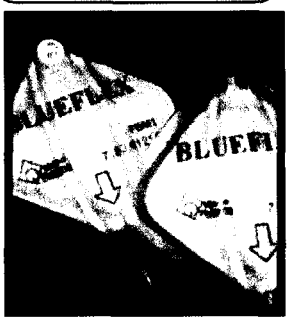
Illumination of plasma + Methylene Blue
(590 nm, 180J/cm^2)

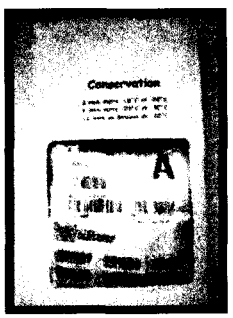
- Intercalation of MB into nucleic acids
- Excitation of MB by visible light
- Oxidation of Guanosine
- Degradation of nucleic acids

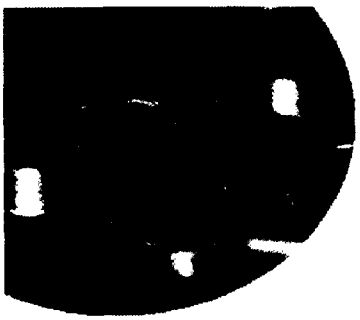
The combined action of Methylene Blue and light is a photodynamic process which blocks transcription and replication of viral RNA and DNA.

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







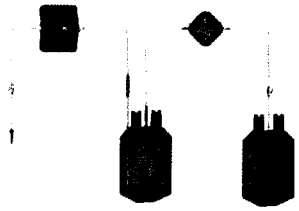
THERAFLEX-MB Plasma

Examples of systems and treatments

Whole Blood Plasma with MB treatment

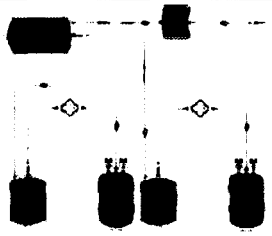
BSV system : plasma filtration with Plasmaflex > MB Pill dissolution > illumination

Whole Blood Plasma with MB treatment and MB removal

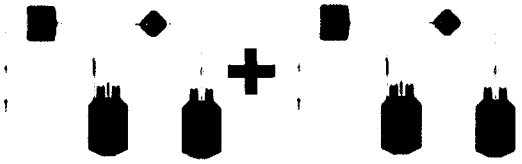


SDV system : plasma filtration with Plasmaflex > MB Pill dissolution > illumination > MB removal with Blueflex

Aphaeresis Plasma : distribution in 2 units

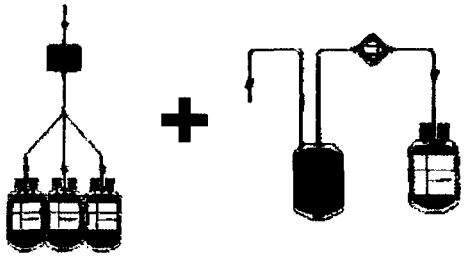


SPV system : filtration of the aphaeresis unit with Plasmaflex MB Pill dissolution and distribution in 2 units > illumination > MB removal with Blueflex



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

Aphaeresis Plasma : distribution in 3 units



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

With 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.[1] However, the appearance of West Nile virus in blood components in the US in 2002, with fatal transmissions in immunocompromised recipients,[2] 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.[3] 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.[3-5] 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 µ*M*, 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 leuko- 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 µ*M*. 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

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.[5-7] Its only application in transfusion has been to achieve virus inactivation of plasma, with prior cell removal by filtration or freeze-thaw lysis[8-10] (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.[3-5,10] 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,[11-14] 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 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,[15] there is no evidence of in vitro inactivation of infectivity at the concentrations used in the transfusion setting.

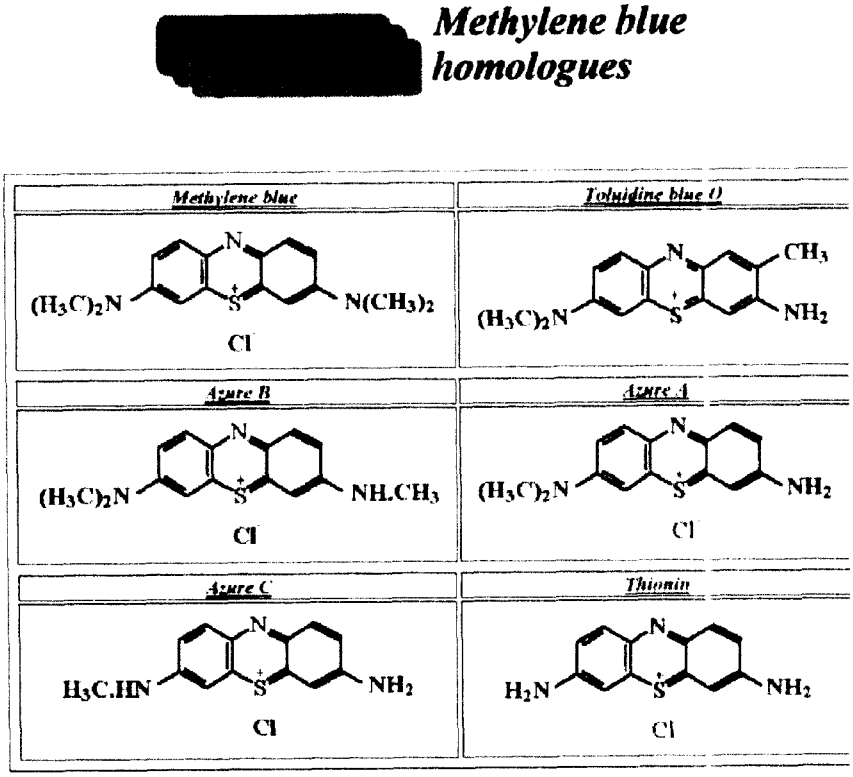

Fig. 1. MB and its photodegradation products.

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

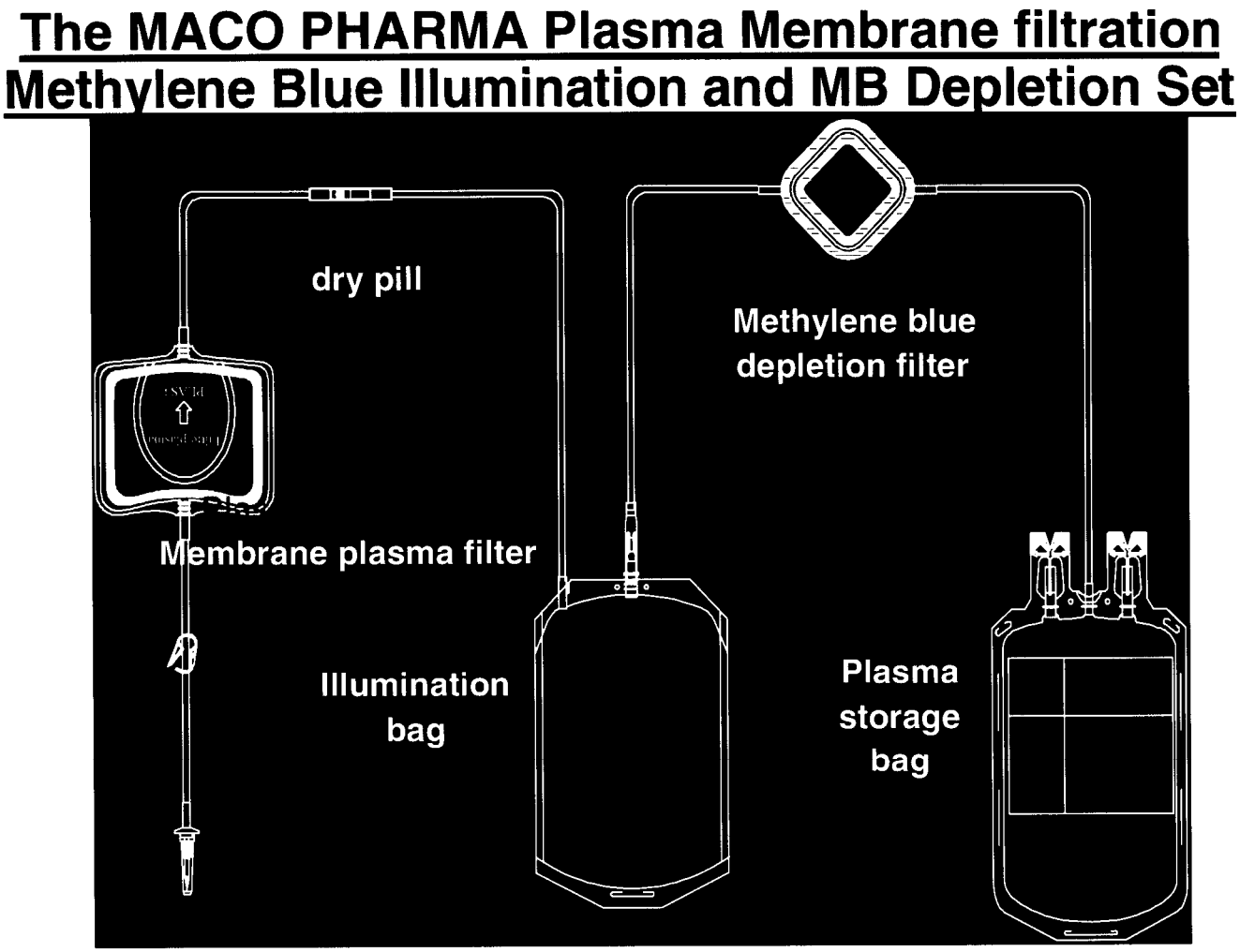

Fig. 2. Schematic representation of the closed bag system for MB treatment of fresh-frozen plasma.

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,[16] 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.[17] The effects on fibrinogen are probably due to an interaction of MB with histidine residues and may result in a modified in vivo clearance.[16,18-20] However, fibrinogen isolated from MB-treated plasma retains normal ability to bind to glycoprotein IIb/IIIa receptors on platelets,[21] an important mechanism in platelet activation and aggregation. The inhibitory effects are ameliorated by the presence of ascorbate[22] but do not appear to result in the formation of any neoantigens[16,18,19] 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).[16,23]

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.[16,24] 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,[10] 24,[23] 39[29]	1.65,[10] 1.80,[16] 2.01,[23] 1.97,[28] 2.05[29]
Fibrinogen (antigen) g/L		2.74[16]
Prothrombin (FII) (U/mL)	↓ 8,[10] 8,[16] 18,[23]	1.15,[10] 1.05,[16] 1.00[23]
FV (U/mL)	↓ 4.5,[10] 21,[16] 32,[23] 10,[28]	0.84,[10] 0.73,[16] 0.79,[23] 0.76[28]
FVII (U/mL)	↓ 8,[10] 9,[16] 7,[23]	1.10,[10] 0.90,[16] 0.90[23]
FVIII (U/mL)	↓ 13,[10] 33,[16] 28,[23] 26,[28] 29[29]	0.78,[10] 0.58,[16] 0.58,[23] 0.83[28]
FIX (U/mL)	↓ 17,[10] 23,[23] 11[28]	1.00,[10] 0.72,[23] 0.88[28]
FX (U/mL)	↓ 13,[10] 7[23]	1.05,[10] 0.90[23]
FXI (U/mL)	↓ 17,[10] 27,[23] 13[28]	1.00,[10] 0.73,[23] 0.84[28]
FXII (U/mL)	↓ 17[10]	1.20[10]
FXIII (U/mL)	↓ 7,[23] 16[29]	1.02,[23] 1.12[29]
vWF antigen (U/mL)	↓ 7,[23] 5[29] →[28]	0.94,[23] 0.83,[29] 1.00[28]
vWF:ristocetin cofactor(U/mL)	↓ 8,[23] 18[29]	0.92,[23] 0.79[29]
C1-inhibitor (U/mL)	↓ 23,[10] →[16]	0.88,[10] 1.03[16]
Antithrombin (U/mL)	↓ 8,[10] 3[23] →[16,23]	0.78,[10] 0.95,[16] 1.00,[23] 0.96[28]
Protein C (U/mL)	→[16,28]	1.03,[16] 0.89[28]
Protein S (U/mL)	→[16]	1.11[16]
α_1-antitrypsin (U/mL)	→[16]	155 mg/dL
Plasminogen (U/mL)	→[10,16]	0.90,[10] 0.98[16]
α_2-antiplasmin (U/mL)	→[16]	0.96[16]

* 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.
‡ [10,16,23]Studies used frozen-thawed plasma.
§ [28,29]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.[25] 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.[26,27] 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.[26]

Levels of thrombin-antithrombin complexes are not elevated in MB-treated plasma,[16] 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.[10,16,23,28] 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,[16] 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,[23,29] but vWF multimeric distribution and cleaving protease activity are reported to be unaffected.[23,28-30]

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.[31]

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[23,32] 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,[23] 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.[32] 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.[33]

Cryosupernatant produced from standard or MB-treated plasma lacks the largest molecular weight forms of vWF.[23] 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,[34] 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,[30] 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, lacate, pH, and glucose compared to standard plasma.[35] 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.[35] This is in contrast to direct treatment of RBCs with MB and light, which results in membrane leakage and enhanced surface binding of IgG.[5-7]

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.[3,4,9,10,20] For comparison, the plasma pathogen-reduction systems described here result in a MB concentration of 1 μM 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,[25,36] this level is reduced approximately $\times 10$, to a final concentration of 0.1 to 0.3 μM. For a 70-kg adult receiving the recommended 15 mL per kg of FFP, this equates to a total MB dose of approximately 33 µg, or less than 1 µg 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[3] (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.[37] 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.[3,5] 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,[3,5] although recently higher doses have been reported as inducing fetal growth retardation.[38] 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 µg per mL (Flament J, Mohr H, and Walker W, written communication, 2000). Wagner et al.[39] has reported genotoxic effects in mouse lymphoma cells at 30 µg per mL of MB, which was enhanced by S9 addition, but failed to detect any activity in vivo in a mouse micronucleus 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[40] 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