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Coagulation factor content of cryoprecipitate prepared from methylene blue plus light virus-inactivated plasma

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Summary. Levels of factor VIII (FVIII) and fibrinogen were assessed in control cryoprecipitate and cryoprecipitate prepared in two centres from plasma subjected to methylene blue (MB) photochemical virus inactivation. The level of coagulation FVIII activity was reduced in plasma by approximately 30% after MB photoinactivation, with only 44% (centre A) and 31% (centre B) of units meeting the current UK specification of 0·7 iu/ml. A revised specification of 0·5 iu/ml is suggested. Losses of less than 11% were seen for von Willebrand factor (VWF)-related activities. Cryoprecipitate prepared from group O or group A MB-treated plasma contained 27–40% less FVIII than control units. This reflected the lower levels in MB-treated plasma. The concentrating power of the cryoprecipitation process was not reduced for FVIII or fibrinogen in MB-treated units. MB cryoprecipitate from centre A still met the UK guideline specification for FVIII and fibrinogen content, whereas at centre B only 62·5% of the group O cryoprecipitates contained > 70 iu FVIII/unit. This may reflect the lower product volume and lower FVIII content of group O plasma used at centre B and suggests that maintenance of total coagulation factor recovery in MB-treated cryoprecipitate will require the higher product volume.

Keywords: virus inactivation, fresh frozen plasma, cryoprecipitate, methylene blue, factor VIII.

Methylene blue (MB), a member of the group of phenothiazine dyes, was shown by Lambrecht *et al* (1991) and Mohr *et al* (1992a) to inactivate viruses in human plasma on exposure to light. The dye binds to and enters via the virus membrane and intercalates with nucleic acids. It then absorbs visible light energy and becomes activated with generation of highly reactive oxygen species. These disrupt the viral membrane and cause destruction of the nucleic acids, particularly at guanosine residues. The resulting nucleic acid modification prevents viral replication. The methylene blue then reverts, in the presence of oxygen, back to its original state. The absorption maximum for methylene blue is 620–670 nm.

Since 1992, the German Red Cross has treated more than 1 500 000 units of plasma with methylene blue (Wieding & Neumeyer, 1992) and the range of viruses inactivated has been well documented, including some non-enveloped viruses as well as many lipid-enveloped ones (Chapman, 1995).

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A disadvantage of using this method is the 20% decrease observed in the levels of fibrinogen and 13% decrease in factor VIII (FVIII) levels in treated plasma (Lambrecht *et al*, 1991). This is probably due to the combination of light, oxygen and MB modifying some histidine residues and other amino acids, thus affecting the functional activities of some proteins (Mohr *et al*, 1992b). Zeiler *et al* (1994) tested plasma treated with MB and compared it with plasma treated with solvent–detergent (SD). The SD method caused less loss of fibrinogen and FVIII, although losses of factors II, V and VII were greater. The SD method can only be used on pools of plasma and requires the SD to be removed after virus inactivation.

Keeling *et al* (1997) prepared cryoprecipitate from SD-treated plasma. The von Willebrand factor (VWF) activity and antigen were 36% and 37% of the control cryoprecipitate values, respectively, and no high molecular weight VWF multimers were seen in either the plasma or the cryoprecipitate. This product therefore would not be suitable for treating people with VWF deficiency. Levels of fibrinogen were acceptable.

The aim of this study was to compare cryoprecipitate

produced from methylene blue plus light virus-inactivated plasma (MB plasma) with cryoprecipitate prepared from control plasma. The Pathinact packs marketed by Baxter were used to prepare MB plasma. Parallel studies were carried out in the UK in Edinburgh (centre A) and in Brentwood (centre B) and were compared.

MATERIALS AND METHODS

Plasma preparation, pooling and aliquoting. In centre A, 64 units of whole blood (32 group O and 32 group A) were collected into NPBI (The Netherlands) top and bottom citrate phosphate dextrose (CPD) blood packs (code C00271117 B). Plasma was produced by centrifugation at 4000 *g* for 20 min. Series of four ABO identical plasma units were pooled to give an approximate volume of 1200 ml. Plasma was selected not to be haemolysed or lipaemic. The pools were mixed for 10 min and then each was divided equally between four transfer packs, each containing 275 ± 10 ml. The residual plasma from each pool was frozen and stored at −40°C for assay of the activation marker prothrombin fragment 1 + 2. Two of the four units from each pool were frozen immediately as controls and the remaining two were subjected to MB treatment as described below.

A similar protocol was used at centre B. However, Baxter CPD top and bottom packs were used.

MB treatment. This was carried out within 8 h of donation. The Pathinact MB system consisted of a disposable unit with integral MB solution (methylthioninii chloridum trihyd 0·097 mg + water to 10 ml) and a white light illumination device. The MB was contained in a PL 732 plastic transfer pack connected to a leucocyte exclusion filter (R7105B). This filter has previously been shown to result in residual leucocyte levels of less than 2×10^3/l (Rider *et al*, 1998). The plasma pack was sterile docked to the disposable unit and then hung up using the total length of tubing to create head pressure. The cannula was broken to allow the plasma to transfer to the PL732 pack through the leucocyte filter. The plasma was mixed with the MB by gently inverting the bag. The MB disposable unit was placed in a Fenwal white light illumination device (4R4006) and the pack illuminated for 30 min (for a dose of 23·6–28·5 J/cm^2). A self-monitoring device provided uniform double-sided illumination of the disposable unit and automatically terminated in the case of lamp failure or door opening, ensuring proper illumination of each unit. When illumination was completed at centre A, the units were blast frozen and stored at below −40°C. At centre B, 10-ml samples were taken from the units and frozen in aliquots at −40°C for assay.

Preparation of cryoprecipitate. At centre A, for each pool (between 24 h and 7 d storage) one control and one MB-treated plasma were thawed in a 4°C waterbath and cryoprecipitates and cryosupernatants were prepared by the fast-thaw method (Prowse & McGill, 1979). These were refrozen and stored at −40°C.

At centre B, fresh frozen plasma was prepared using a blast freezer and stored at −40°C overnight. A slow thawing technique at 4°C overnight was used to prepare cryoprecipitate which was then refrozen at −40°C. Only group O units were processed to cryoprecipitate at this centre as they represent the worst possible case in terms of the starting levels of FVIII.

Within 7 d of preparation, all plasma and cryoprecipitates together with six cryosupernatants (three group A, three group O from centre A and six group O from centre B) were thawed at 37°C and aliquoted. Factor VIII and ristocetin cofactor (RiCoF) activity assays were performed immediately. Aliquots (2 ml) were stored at −40°C for von Willebrand factor antigen (VWF:Ag) and fibrinogen assays, which were performed within 4 weeks of freezing. VWF multimer analysis was performed on six pools each of cryoprecipitates and cryosupernatants.

Assays. Prothrombin fragment 1 + 2 was assayed using the Behring Diagnostics enzyme-linked immunosorbent assay (ELISA) kit. Fibrinogen was measured using the Clauss (centre A) or prothrombin time-derived method (centre B). A one-stage automated clot detection system (Coag-a-mate X2, Organon Teknika, at centre A and ACL-100 at centre B) was used for the FVIII and FXI assays. RiCoF activity was assayed using freeze-dried platelets by a method based on that of Macfarlane *et al* (1975) and VWF:Ag was assayed by ELISA using Dako antibodies to human VWF (centre A) or a Stago VWF:Ag ELISA kit (centre B). von Willebrand multimers were analysed using the PHAST system and enhanced chemiluminescent (ECL) blotting (Lawrie *et al*, 1990).

Statistics. Results are presented as means ± standard deviation. Statistical comparison was performed by the Student paired *t*-test.

RESULTS

Plasma

The treatment of single-donor plasma with methylene blue in the presence of light using the Pathinact system inactivated a range of viruses but resulted in a loss of some coagulation factor activity (Table I). Relative to paired samples of untreated plasma, these losses were significant ($P < 0·001$) for fibrinogen (21%) and factor XI (42%) at both test sites. The loss of 5% VWF:Ag at centre A was also significant. More importantly, a 34% loss of coagulation FVIII, the parameter usually used to assess plasma quality, was observed at both testing sites. Overall 44% (centre A) and 31% (centre B) of MB-treated units contained > 0·7 iu FVIII/ml (overall mean 0·67, range 0·43–0·88), whereas all control units (mean 1·01, range 0·76–1·37 iu/ml) met this standard. There was no evidence of any activation during plasma preparation with prothrombin fragment 1 + 2 levels remaining within the normal range.

Cryoprecipitate

Recovery was defined as total factor content per unit of cryoprecipitate expressed as a percentage of total content in the corresponding plasma unit. Further processing of control and treated plasma to cryoprecipitate and cryosupernatant was also assessed by slightly different processes

Table I. Coagulation factors in control and MB-treated plasma.

	Control plasma (mean ± SD)	MB plasma (mean ± SD)	Per cent loss due to MB treatment
Volume (ml)	277 ± 7	278 ± 7	
	271 ± 6	262 ± 13	
FVIII (iu/ml) mean	1·01 ± 0·17	0·69 ± 0·12***	32
	1·01 ± 0·14	0·65 ± 0·14***	36
% with > 0·70 iu/ml FVIII	100	44	
	100	31	
FVIII group O (iu/ml)	0·89 ± 0·08	0·61 ± 0·08***	32
	0·94 ± 0·11	0·55 ± 0·08***	41
FVIII group A (iu/ml)	1·13 ± 0·15	0·77 ± 0·08***	32
	1·08 ± 0·13	0·74 ± 0·11***	32
RiCoF (iu/ml)	0·98 ± 0·19	0·92 ± 0·22	6
(Group A only for centre B)	1·01 ± 0·17	0·90 ± 0·21***	11
VWF:Ag (iu/ml)	0·92 ± 0·20	0·87 ± 0·20***	5
	0·99 ± 0·31	0·96 ± 0·30	3
Fibrinogen (g/l)	2·53 ± 0·17	1·87 ± 0·16***	26
	2·91 ± 0·25	2·48 ± 0·29***	15
FXI (iu/ml)	0·94 ± 0·07	0·57 ± 0·07***	39
(Group O only for centre B)	0·80 ± 0·10	0·42 ± 0·07***	47
Prothrombin fragment 1 + 2 (nM)	0·61 ± 0·19	Only pool assayed	
	0·78 ± 0·26		

Centre A results are shown first followed by centre B results with $n = 16$ (eight group O and eight group A).
*$P < 0·05$, **$P < 0·01$, ***$P < 0·001$ compared with controls.

on the two sites. The recovery of coagulation factors from MB and control plasma was similar and did not differ at the two test sites except in the case of VWF:Ag (Table II). Overall, mean recoveries from centres A and B for control and MB plasma were 49% and 50% for FVIII and 37% and 29% ($P < 0·001$) for fibrinogen. A lower recovery of VWF:Ag at centre B (75% and 79%) than centre A (106% and 97%) may relate to the lower residual volume for cryoprecipitate at centre B. Multimeric analysis of VWF in cryoprecipitate prepared at centre A revealed no difference between product from control and MB-treated plasma (Fig 1).

Table II shows the results obtained for cryoprecipitate expressed as content per unit. The significantly lower content of coagulation factors in the MB-treated products presumably reflects the losses resulting from MB treatment of plasma, given the similar recoveries obtained during subsequent processing to cryoprecipitate.

Cryoprecipitate prepared from MB-treated plasma at centre A met the requirement that at least 75% of units contain 140 mg fibrinogen and 70 iu FVIII (Table II). This was not true for the FVIII content of units from centre B, where cryoprecipitate was only prepared from group O plasma, which has a lower starting FVIII plasma content (Table I).

The ability of the cryoprecipitation process to concentrate coagulation factors of interest was apparently higher at centre B than at centre A. By normalizing the unit content of product to a nominal volume of 30 ml, this difference in product potency was shown to be due to the different cryoprecipitate product volumes (Table III).

Selected units of cryosupernatant plasma were also assayed. The results presented in Table IV demonstrate that some 25% of FVIII in plasma was not accounted for and was lost during the cryoprecipitation process.

DISCUSSION

The Pathinact system used for MB treatment in this study is based on a system developed by the German Red Cross (Mohr *et al*, 1992a), but allows a reduced process time of 30 min as both sides of the pack are illuminated. Leucodepletion to levels below 2×10^3 leucocytes/l (Rider *et al*, 1998) was also achieved by filtration rather than a freeze–thaw step. The amount of methylene blue used was 83 μg per unit, which is much less than amounts used without adverse effect in other therapeutic procedures.

Coagulation factor losses of 13% FVIII, 20% fibrinogen and 17% FXI were reported for the German Red Cross system (Lambrecht *et al*, 1991), but higher losses of 34%, 20% and 43%, respectively, were found for the Pathinact system in the current study. This resulted in the fact that MB-treated plasma did not meet the current UK specification that 75% of units contain at least 0·7 iu FVIII/ml. On the basis of a further study (unpublished observation), in which 75% of 169 MB plasma units (prepared in Newcastle, North London and Edinburgh, UK) contained at least 0·56 iu/ml, a draft specification for this product that 75% of units should contain more than 0·5 iu/ml has been proposed (unpublished observations).

Castro *et al* (1998) measured various clotting factors in cryoprecipitate prepared from MB-treated plasma. The losses compared with control cryoprecipitate were FVIII 38%,

Table II. Coagulation factors in cryoprecipitate prepared from control and MB plasma.

Total amount per unit	Control cryoprecipitate	MB cryoprecipitate	Per cent loss due to MB treatment	Recovery from control plasma†	Recovery from MB plasma†
Volume (ml)	40 ± 7	42 ± 12			
	16 ± 4	26 ± 6			
FVIII group O (iu)	126 ± 14	75 ± 11***	40	51	44
	106 ± 14	77 ± 12***	27	42	54
FVIII group A (iu)	171 ± 22	106 ± 17***	38	55	50
(no centre B study)					
FVIII (iu) mean (centre A only)	149 ± 29	91 ± 21***	39	53	47
% units with > 70 iu FVIII	100	88			
	100 (group O)	62·5 (group O)			
RiCoF (iu)	212 ± 47	170 ± 51***	20	78	66
	142 ± 22	148 ± 27	0	NA	NA
VWF:Ag (iu)	271 ± 62	233 ± 48***	14	106	97
	155 ± 22	159 ± 41	0	75	79
Fibrinogen (mg)	271 ± 31	159 ± 22***	41	39	31
	288 ± 82	177 ± 53***	39	36	27
% units with > 140 mg fibrinogen	100	88			
	88	75			

For centre A results (shown first), $n = 16$ (eight group O, eight group A); for centre B, $n = 8$ (group O only).
*$P < 0{\cdot}05$. **$P < 0{\cdot}01$. ***$P < 0{\cdot}001$ compared with controls.
†Total factor content per unit cryoprecipitate expressed as percentage of total content in the corresponding plasma unit.

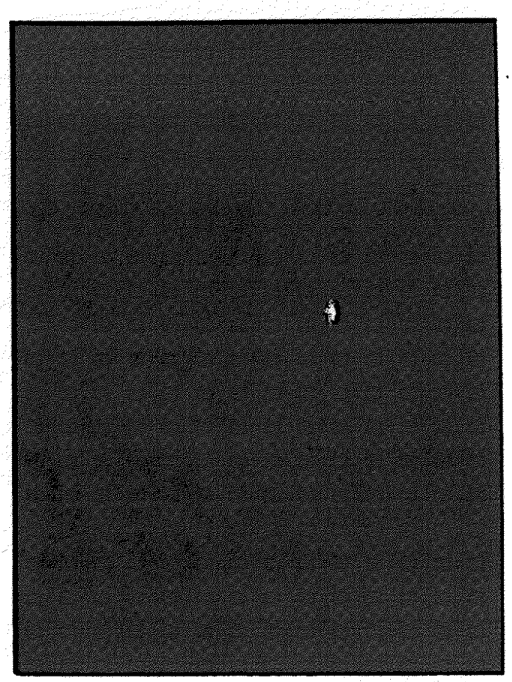

Fig 1. VWF multimers. Tracks 1–6 (left to right) show plasma standard, control cryoprecipitate, control cryosupernatant, MB cryoprecipitate, MB cryosupernatant and plasma standard respectively.

VWF activity 25%, VWF:Ag 24% and fibrinogen 55%. Similar or lower losses were seen in this study.

When both group A and group O units of MB plasma were processed to cryoprecipitate at centre A, the mean results for the product (Table II) did meet the UK specification (Guidelines for the Blood Transfusion Services in the United Kingdom, 1996) that 75% of units contain at least 140 mg fibrinogen and 70 iu FVIII. The lower results obtained at centre B were explained by the exclusive use of group O units.

As it is prepared from single donations, MB plasma is not required to have a drug licence or to be subject to formal assessment of clinical efficacy under UK regulations. Most frozen plasma is used for the replacement of vitamin K-dependent clotting factors in liver disease. A formal trial comparing solvent–detergent plasma, containing a mean 0·55 iu FVIII/ml, with standard plasma in this indication did not reveal any difference in prothrombin time or activated partial thromboplastin time correction (Williamson *et al*, 1999). Based on the *in vitro* results obtained in this study, a different outcome for MB plasma would not be expected. Neither SD nor MB plasma has been subject to trial in patients with multiple coagulation deficiencies, such as may be observed during disseminated intravascular coagulations.

Cryoprecipitate is now largely prescribed in the UK as a source of fibrinogen. The MB cryoprecipitate characterized in this study had a lower FVIII and fibrinogen content than reported for product from SD plasma (Keeling *et al*, 1997),

Table III. Comparison of coagulation factors in control and MB plasma and cryoprecipitate, and of the efficacy of the cryoprecipitation process.

Coagulation factor concentration	Plasma	Cryoprecipitate	Fold increase (normalized)
FVIII (iu/ml)			
Control	1·01	3·89	3·9 (5·2)
	1·01	7·08	7·0 (3·7)
MB treated	0·69	2·35	3·4 (4·8)
	0·65	3·13	4·8 (4·2)
RiCoF (iu/ml)			
Control	0·98	5·52	5·6 (7·5)
	NA	9·31	NA
MB treated	0·92	4·36	4·7 (6·6)
	NA	6·12	NA
VWF:Ag (iu/ml)			
Control	0·92	7·15	7·8 (10·4)
	0·76	10·13	13·3 (7·1)
MB treated	0·87	6·07	7·0 (9·8)
	0·77	6·54	8·5 (7·4)
Fibrinogen (mg/ml)			
Control	2·53	6·94	2·7 (3·6)
	2·93	19·00	6·5 (3·5)
MB treated	1·87	4·09	2·2 (3·1)
	2·52	7·43	2·9 (2·6)

The fold increase figure was calculated by dividing the mean cryoprecipitate concentration for each factor by the mean plasma concentration. Centre A results are followed by those from centre B.

'Normalized' values are calculated for the same total factor recovery suspended in a nominal 30 ml cryoprecipitate volume.

Table IV. Coagulation factors in cryosupernatant prepared from control and MB-treated units.

Total amount per unit	Control cryosupernatant	MB cryosupernatant
Volume (ml)	237 ± 8	226 ± 8
	235 ± 10	223 ± 9
FVIII (iu)	47 ± 7	38 ± 8
	47 ± 19	33 ± 8
RiCoF (iu)	< 15	< 15
	26 ± 5	28 ± 4
VWF:Ag (iu)	36 ± 9	39 ± 7
	42 ± 9	47 ± 5
Fibrinogen (mg)	423 ± 29	314 ± 28
	476 ± 56	410 ± 69

Centre A results shown first, $n = 6$ (three group O, three group A); for centre B, $n = 8$ (group O only); results are given as total amounts per unit.

but a higher RiCoF and VWF:Ag level together with the retention of high molecular weight VWF multimers.

The reductions in FVIII and fibrinogen levels may necessitate dose adjustment for both plasma and cryoprecipitate, but definitive evidence of this can only emerge from ongoing clinical assessment or formal clinical trial.

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