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Manufacture and in vitro Characterization of a Solvent/Detergent-Treated Human Plasma

Abstract

We have developed a modified solvent/detergent (S/D) treatment to inactivate viruses in human plasma using 1% w/w final concentrations of tri(n-butyl) phosphate (TNBP) and Triton X-100 and an incubation period of 4 h at 30°C. The procedure inactivates $\geq 10^6$ chimpanzee-infectious doses (CID₅₀) of HBV, $\geq 10^5$ CID₅₀ of HCV, and $\geq 10^{6.2}$ tissue culture infectious doses (TCID₅₀) of HIV. After virus inactivation, eleven plasma batches were lyophilized and 12 batches were deep-frozen until further use. The batches were characterized by extensive laboratory tests including measurement of clotting factors I-XIII, von Willebrand factor, plasminogen, inhibitors of blood coagulation and fibrinolysis, and other clinically important plasma proteins. All parameters were determined before and after S/D treatment. Twelve conventional single donor plasma units served as control. There were no marked losses of activities of clotting factors, antithrombin III, protein C, plasminogen, and C1-esterase inhibitor due to treatment. After the S/D step, the levels of these parameters were within the normal range in all batches. The same holds true for total protein, immunoglobulins, albumin, complement factors C3 and C4, haptoglobin, hemopexin, caeruloplasmin, α_1 -antitrypsin, and pH. Protein S and α_2 -antiplasmin activities decreased by about 50% and were frequently found to be slightly below the lower limit of the respective normal range after treatment. The interindividual variations of all proteins analysed were significantly lower than in the single donor plasma units. The S/D procedure did not lead to increases of markers indicating activation of hemostasis. We conclude that lipid-enveloped viruses can be inactivated by the S/D procedure described in this study without critical reduction of recoveries of plasma proteins.

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Introduction

A series of effectively virus-inactivated plasma derivatives are presently available to treat volume deficiency, inherited and acquired deficiencies of coagulation factors and inhibitors as well as immunoglobulin deficiencies [1]. Several procedures for virucidal treatment of plasma derivatives have been described, including heating in the lyophilized state [2], wet heating (pasteurization) [3-5], heating with hot vapour [6], treatment with beta-propiolactone and ultraviolet irradiation [7], and treatment with the organic solvent tri(n-butyl)phosphate (TNBP) in combination with a detergent [8, 9]. These have recently been reviewed [10-12]. In combination with careful donor selection and donor blood screening, these procedures reduce the risk of transmitting virus diseases by plasma derivatives to a very low level. Apart from the fact that plasma is a scarce and valuable raw material for producing its derivatives, fresh frozen plasma (FFP) is still needed for treatment of dilutional coagulopathy in massively transfused patients, DIC, loss coagulopathy, 'complex coagulopathies', e.g. liver disease combined with DIC or overt bleeding, for massive plasma exchange, and for treating inherited deficiencies of factors V and XI, and thrombotic thrombocytopenic purpura.

Plasma carries a risk of virus transmission similar to that of whole blood. As reported recently [13], three modifications of solvent/detergent treatment appear to be able to inactivate hepatitis viruses and human immunodeficiency viruses sufficiently while comprehensively retaining the biological activities of the plasma proteins.

This article describes the production of lyophilized and deep-frozen batches of human plasma using an improved TNBP/detergent procedure and the *in vitro* characterization of this virus-inactivated plasma.

Materials and Methods

Processing and Virus Inactivation of Human Plasma

Plasma units for producing lyophilized solvent/detergent (S/D)-treated and deep-frozen batches were provided from Octapharma Corp., Glarus, Switzerland, and from the Red Cross Center Hagen, FRG, respectively.

Two hundred and fifty fresh-frozen plasma units (each 200-250 ml) of identical blood group that had been stored deep frozen at at least -30°C were thawed at 30°C using an appropriately equipped vessel made of stainless steel. Plasma units, which were distinctly identified being haemolysed or lipaemic, were removed prior to pooling and thawing. After complete thawing and cooling to 25°C, the pH was adjusted to 7.0-7.3 by addition of 5 mmol/l NaH_2PO_4 and subsequently controlled by flooding with carbon dioxide (CO_2) during

the entire process. For this purpose, the surface of the plasma in the reaction vessel was steadily kept under a layer of CO_2 by tightly applying the end piece of a gas tube to the plasma surface, without dipping it in, however. The gas supply was regulated to a rate of 3-5 litres/min as long as the pH did not drop below a value of 7.1.

In order to remove small-sized particles which are able to trap and protect virions against the inactivation treatment, the plasma pool was filtered by means of a 1- μm filter-cartridge at room temperature. Subsequently, tri(n-butyl)phosphate (TNBP) and Triton X-100 were added to a final concentration of 1% w/w each. The reagents were purchased from Merck, Darmstadt, FRG, and from Serva, Heidelberg, FRG, and were of analytical grade. The mixture was heated to 30°C for 30 min and pumped to a second vessel separately prepared in a specially equipped virus-free area. We took this precaution in order to avoid any risk of recontamination by viruses. The plasma/TNBP/Triton X-100 mixture was subsequently kept at 30°C for 4 h under slow stirring.

After completion of the inactivation process, 5% v/v ricinus oil (Neuber, Vienna; Eur. pharm. grade) was added under moderate stirring for 45 min. Then the stirring was stopped and the mixture left to stand for 30 min in order to assure a complete separation of the oil and plasma layers. The plasma layer was withdrawn and cleared by a cascade of filters using 1- and 0.45- μm pore sizes. Thereafter, the clear plasma was passed over an octadecyl (C-18) resin supplied by Waters Comp., Eschborn, FRG (0.2 litres of resin per litre of plasma).

TNBP was measured by a particularly designed gaschromatographical assay using a gas-chromatographic system Hewlett-Packard, Type 5890 A and capillar columns HP 1.5 M, 0.53 mm. The internal standard was tri-n-pentylphosphate (Merck) and the injected sample volume was 2 μl . The sample to be injected was obtained by a liquid-liquid extraction of the plasma samples using hexane (Merck, analytical grade). The detection limit was 0.5 $\mu\text{g/ml}$.

Triton X-100 was determined by a chromatographic assay on a HPLC system, specially tailored to this purpose: HPLC system Knauer Berlin, FRG, separation column C-8 Polygosil, 4 \times 125 mm Knauer Berlin, FRG, Triton X-100 pure grade Serva, Heidelberg, FRG, injected volume 200 μl . The sample to be injected was obtained after the Triton X-100 content of the plasma sample had been concentrated on a C-18 column (Waters, Eschborn, FRG, No. 51910) under reversed phase chromatographic conditions. The detection limit was 2.0 $\mu\text{g/ml}$. Following this treatment, the residual concentrations of TNBP and Triton X-100 dropped to less than 0.5 and 2 $\mu\text{g/ml}$, respectively.

An inevitable accompanying phenomenon of the total process is a dilution of the plasma pool by about 10%. The virus-inactivated plasma was filled either in plastic blood bags to be stored at -30°C or lower or in infusion bottles which were spin frozen and subsequently lyophilized until further use.

Material for in vitro Characterization

We examined 11 batches of lyophilized and 12 batches of deep-frozen plasma before and after S/D treatment. Twelve single donor plasma units of blood group A served as control. Before S/D treatment 10-ml aliquots of each lot were deep frozen at -70°C. Virus-inactivated deep-frozen plasma units were thawed at 37°C and lyophilized units were reconstituted with twice distilled water. The screening tests of blood coagulation and clotting factor activities were determined immediately. The remaining parameters had to be measured after renewed freezing and thawing. For this purpose, 10-ml

Table 1. Screening tests of blood coagulation and clotting factor activities in the 11 lyophilized and the 12 deep-frozen batches before and after S/D treatment

Parameters	Normal range	Lyophilized plasma units (n = 11)		decrease or increase %
		median (min-max)		
		before S/D	after S/D	
PT, s	12.5-16.1	13.1 (12.5-14.5)	14.1 (12.8-15.0)	7.6***
APTT, s	28-41	34.0 (30-39)	39.0 (34-41)	14.7***
TT, s	14-20	17.0 (14-17)	16.0 (15-18)	5.8
Fibrinogen, g/l	1.45-3.85	2.0 (1.65-2.3)	1.70 (1.55-2.10)	15.0***
Factor II, U/ml	0.65-1.54	0.90 (0.84-0.95)	0.85 (0.70-0.90)	5.5*
Factor V, U/ml	0.54-1.45	0.90 (0.70-1.0)	0.75 (0.60-0.85)	16.6***
Factor VII, U/ml	0.62-1.65	0.90 (0.68-1.04)	0.85 (0.70-0.90)	5.5
Factor X, U/ml	0.68-1.48	0.90 (0.84-1.04)	0.85 (0.80-1.05)	5.5
Factor VIII-1, U/ml	0.45-1.68	0.89 (0.65-1.15)	0.65 (0.50-0.90)	26.9*
Factor VIII-2, U/ml	0.52-1.55	0.95 (0.65-1.56)	0.75 (0.55-1.15)	21.0*
Factor IX, U/ml	0.45-1.48	1.00 (0.75-1.10)	0.75 (0.65-0.85)	25.0*
Factor XI, U/ml	0.42-1.44	0.90 (0.70-1.15)	0.65 (0.50-1.05)	27.7
Factor XII, U/ml	0.40-1.52	0.95 (0.80-1.10)	0.80 (0.65-0.90)	16.6***
Factor XIII, U/ml	0.65-1.65	1.00 (0.70-1.40)	0.80 (0.70-1.20)	20.0*
vWF (Rcof), U/ml	0.45-1.75	0.95 (0.60-1.20)	0.75 (0.50-1.20)	21.0

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$. The normal ranges (2.5 and 97.5 percentiles), medians, minimum and maximum values and percentages of decrease or increase are given. Factor VIII-1 = Factor VIII one-stage assay; factor VIII-2 = factor VIII two stage assay.

aliquots were deep frozen and kept at -70°C until further use. Two of the 11 lyophilized and 5 of the 12 deep-frozen batches were blood group O plasmas. This has to be considered when analyzing factor VIII (FVIII) and von Willebrand factor (vWF), since blood group O donors have significantly lower FVIII and vWF levels compared to blood group A, B, or AB donors [14].

Assays

The normal ranges were derived from the plasmas of 100 healthy individuals for all parameters of coagulation and fibrinolysis and for other plasma proteins except for some activation products of blood coagulation. Blood was drawn using a 10-gauge butterfly needle. Platelet-poor plasma was obtained by mixing 9 parts blood and 1 part 0.109 mol/l sodium citrate and centrifugation at 2,000 g for 15 min. Platelet-poor plasma was snap frozen at -70°C . In order to establish representative normal ranges for prothrombin split products (FII splits) and for thrombin/antithrombin III complexes (TAT), 20 conventionally produced fresh-frozen plasma units containing the acid citrate preservative solution CPD-A₁ were thawed, deep-frozen again and rethawed before analysis. The 2.5 and 97.5 percentiles were used to define the reference ranges.

All parameters were determined in duplicate. Prothrombin time (PT), activated partial thromboplastin time (APTT), thrombin time (TT), and clottable fibrinogen were measured using reagents from Boehringer, Mannheim, FRG, and a coagulation analyzer KC10A from Amelung, Lemgo, FRG. Factors II, V, VII, X, VIII, IX, XI, and XII were determined by one-stage clotting assays using human deficient plasmas from Immuno, Heidelberg, according to the manu-

facturer's instructions. Factor VIII was also determined by a two-stage clotting assay by means of a test kit from the same company and by a chromogenic substrate assay (Coatest factor VIII) from Kabi Diagnostika, Freiburg, FRG. Factor XIII was assayed according to Bohn and Haupt [15] and von Willebrand factor (vWF) was determined as ristocetin cofactor activity (Rcof) according to MacFarlane and Zucker [16] using test kits from Behringwerke, Marburg, FRG.

We quantified antithrombin III (AT III), protein C, α_2 -antiplasmin, plasminogen, and C1-esterase inhibitor (C1-INH) by chromogenic substrate assays from Boehringer, Mannheim, Immuno, Heidelberg, and Instrumentation Laboratory, Kirchheim, respectively. Protein S activity was measured according to Preda et al. [17] using a test kit from Instrumentation Laboratory, Kirchheim. Protein S concentration was measured by electroimmunodiffusion using reagents from Immuno, Heidelberg. We used enzyme immunoassays from Behringwerke to determine TAT and FII splits and an enzyme immunoassay from Baxter, Unterschleissheim, to quantify D-dimers. Fibrin monomers were measured according to Largo et al. [18] using reagents from Boehringer. Thrombin-, factor Xa-, and kallikrein-like activities were determined using the chromogenic substrates S-2238, S-2222, and S-2302 from Kabi Diagnostika, Freiburg [19].

We assayed immunoglobulins G, A, and M, albumin, complement factors C3 and C4, α_1 -antitrypsin, haptoglobin, hemopexin, caeruloplasmin, and α_2 -antiplasmin concentration by kinetic nephelometry using a nephelometer and reagents from Behringwerke. Lipoprotein(a) was measured by the same equipment using antibodies and standards from Immuno. Total protein, triglycerides, cholesterol, and pH were quantified by standard procedures.

Deep-frozen plasma units (n = 12)				decrease or increase %
median (min-max)				
before S/D		after S/D		
12.5	(12.5-14.2)	12.8	(12.0-14.7)	2.4
30.5	(29-32)	32.5	(30-37)	6.5*
17.0	(16-18)	16.0	(16-17)	5.8
2.07	(1.85-2.15)	2.07	(1.85-2.10)	0.0
0.90	(0.88-0.96)	0.88	(0.84-0.96)	2.2
0.90	(0.80-1.0)	0.85	(0.75-1.0)	5.5
0.88	(0.80-1.20)	0.92	(0.80-1.20)	4.5
1.02	(0.98-1.15)	1.00	(0.95-1.15)	1.9
0.85	(0.70-1.20)	0.65	(0.48-1.20)	23.5***
0.87	(0.50-1.40)	0.85	(0.70-1.05)	2.2
0.77	(0.70-0.90)	0.75	(0.45-0.90)	2.5*
0.97	(0.85-1.20)	0.80	(0.45-1.10)	17.5**
0.83	(0.70-1.06)	0.80	(0.70-1.10)	3.6
1.10	(0.90-1.40)	0.95	(0.90-1.30)	13.6
0.96	(0.74-1.30)	0.90	(0.60-1.05)	6.2

Statistical Analysis

Wilcoxon matched pairs signed rank test was used to compare results obtained before and after virus inactivation. The Mann-Whitney U test was used to test for differences between results obtained from the three groups of plasmas (lyophilized and deep-frozen virus-inactivated plasma and conventional single donor plasma).

Results

Screening Tests of Blood Coagulation and Clotting Factor Activities

The time until complete solution of the lyophilized plasmas took between 8 and 14 min (mean: 10 min), which was comparable to the time needed for complete thawing of the deep frozen bags in a water bath. In all lyophilized and deep-frozen batches, the screening tests of blood coagulation and clotting factor activities were within the normal range before and after virus inactivation. The median values, percentages of decrease or increase and significance levels are presented in table 1. The values obtained after S/D treatment are not corrected due to dilution. With the exception of fibrinogen in the deep-frozen plasma units, all clotting factors declined to different de-

grees. Factors VIII and XI activities dropped most distinctly. There were no significant differences between the factor VIII values measured by one-stage assay, two-stage assay, and chromogenic substrate assay, respectively (the chromogenic substrate assay results are not shown in table 1). The two lowest factor VIII and vWF levels were found in blood group 0 plasmas. However, in the two lyophilized and five deep-frozen blood group 0 batches, factor VIII and vWF levels were not significantly lower compared to plasmas from blood group A, B or AB. On the whole, clotting factor activities decreased more markedly in the lyophilized plasmas than in the deep-frozen batches. The batch-to-batch variations of all clotting factor levels in the pooled plasmas before as well as after S/D treatment were significantly lower than the bag-to-bag variations in the 12 conventionally produced single donor plasma units (data not shown).

Inhibitor Activities, Plasminogen, and Markers of Activation of Coagulation and Fibrinolysis

The median plasma levels before and after treatment, percentages of decrease, and levels of significance are shown in table 2. Despite decreases to different degrees, AT III, protein C, plasminogen, and C1-INH activities remained within the normal range after S/D treatment. Protein S and α_2 -antiplasmin activities markedly declined due to the virus inactivation procedure. After treatment, protein S activities were diminished in all lyophilized lots (minimum: 0.45 U/ml) and in 6 of the 12 deep-frozen plasmas (minimum: 0.52 U/ml). α_2 -Antiplasmin levels were below the normal range in all lyophilized and in 7 of the 12 deep-frozen virus-inactivated lots, the lowest plasma levels being 0.40 and 0.52 U/ml, respectively. Protein S concentration was not affected by treatment, whereas α_2 -antiplasmin concentration dropped by 43% on average (data not shown). The batch-to-batch variations of all inhibitor activities and of plasminogen were significantly lower in all pooled plasmas before and after S/D treatment than in the 12 single donor units serving as control.

Both TAT and FII splits were within the normal range in all lyophilized batches. Non increased thrombin-, factor Xa-, and kallikrein-like activities could be found compared to the values measured in conventional FFP units or in native plasma obtained immediately after blood collection in healthy individuals. TAT levels were elevated in 3 deep-frozen plasmas before and in 1 deep-frozen plasma after treatment. In 2 treated plasmas, FII splits were also increased. On the whole, the virus inactivation process led to a significant decrease of TAT in all treated plasmas and to an increase of FII splits, which was significant in the

Table 2. Inhibitors of haemostasis, plasminogen and C1-esterase inhibitor activities, levels of thrombin-antithrombin-III complexes, prothrombin splits and fibrin monomers in the 11 lyophilized and the 12 deep-frozen batches before and after S/D treatment

Parameters	Normal range	Lyophilized plasma units (n = 11)		decrease or increase %
		median (min-max)		
		before S/D	after S/D	
Antithrombin III, U/ml	0.72-1.45	0.99 (0.85-1.10)	0.90 (0.75-1.05)	9.0*
Protein C, U/ml	0.58-1.64	0.90 (0.84-0.99)	0.82 (0.67-0.90)	8.8***
Protein S, U/ml	0.56-1.68	0.95 (0.85-1.26)	0.61 (0.41-0.63)	35.7***
Plasminogen, U/ml	0.68-1.44	0.95 (0.83-0.99)	0.80 (0.70-0.91)	16.6***
α_2 -Antiplasmin, U/ml	0.72-1.32	0.94 (0.85-0.99)	0.44 (0.39-0.60)	53.1***
C1-esterase inhibitor, U/ml	0.60-1.24	0.85 (0.75-0.95)	0.65 (0.60-0.81)	23.5***
TAT, μ g/l	1.00-50	3.50 (1.90-30.0)	2.10 (1.30-7.0)	40.0***
FII splits, nmol/l	0-2.0	0.40 (0.24-0.65)	0.56 (0.23-0.80)	40.0
Fibrin monomers, μ g/ml		< 15	< 15	

* p < 0.05; ** p < 0.01; *** p < 0.005. The normal ranges (2.5 and 97.5 percentiles), medians, minimum and maximum values and percentages of decrease are given.

Table 3. Total protein, immunoglobulins, albumin, complement factors C3 and C4, haptoglobin, hemopexin, caeruloplasmin, α_1 -antitrypsin and pH in the 11 lyophilized and the 12 deep-frozen batches before and after S/D treatment

Parameters	Normal range	Lyophilized plasma units (n = 11)		decrease or increase %
		median (min-max)		
		before S/D	after S/D	
Total protein, g/l	48-64	56 (5.20-6.20)	52 (4.9-5.9)	7.1
IgG, mg/dl	660-1,450	844 (750-1,090)	749 (670-1,090)	11.2*
IgA, mg/dl	75-420	193 (175-240)	164 (145-200)	15.0***
IgM, mg/dl	40-310	145 (115-215)	103 (90-125)	28.9***
Albumin, mg/dl	2,790-4,050	3,240 (2,980-3,690)	2,970 (2,710-3,370)	8.3***
C3, mg/dl	40-72	65 (60-70)	53 (50-60)	18.4***
C4, mg/dl	12-34	24 (17-30)	19 (16-21)	20.8***
α_1 -Antitrypsin, mg/dl	110-260	214 (190-255)	190 (170-225)	11.2*
Haptoglobin, mg/dl	55-235	142 (130-160)	119 (110-140)	16.1***
Hemopexin, mg/dl	38-95	72 (70-90)	59 (52-70)	18.0***
Caeruloplasmin, mg/dl	15-40	24 (20-33)	18 (17-22)	25.0***
pH		7.2 (7.0-7.40)	7.0 (6.50-7.60)	2.7

* p < 0.05; ** p < 0.01; *** p < 0.005. The normal ranges (2.5 and 97.5 percentiles), medians, minimum and maximum values and percentages of decrease are given.

deep-frozen batches (table 2). Fibrin monomers were below the detection limit, and D-dimers were in the normal range or not detectable in the 2 deep-frozen and the 2 lyophilized lots examined for these parameters. Further evidence that the virus inactivation procedure does not lead to activation of hemostasis are the ratios of FVIII

activities measured by one-stage and two-stage assay, which were well below 2.0 (table 1). It is well established that FVIII activated by thrombin yields markedly increased FVIII activities when using the one-stage assay, whereas the two-stage assay results are not influenced by activated FVIII [20-22].

Deep-frozen plasma units (n = 12)				
median (min-max)				decrease or increase %
before S/D		after S/D		
1.02	(0.86-1.10)	0.93	(0.81-1.10)	8.8
0.88	(0.81-1.20)	0.77	(0.60-0.86)	12.5
1.11	(0.80-1.20)	0.60	(0.35-0.81)	45.9***
0.95	(0.90-1.05)	0.88	(0.85-0.98)	7.3***
1.01	(0.89-1.09)	0.58	(0.48-0.64)	42.5***
0.82	(0.77-0.87)	0.75	(0.66-0.82)	8.5***
11.5	(0.30-98.0)	6.40	(2.00-59.0)	44.3**
0.33	(0.21-1.70)	1.15	(0.33-3.40)	248 **
<15		<15		

Deep-frozen plasma units (n = 12)				
median (min-max)				decrease or increase %
before S/D		after S/D		
56	(5.40-6.40)	54	(5.0-6.10)	8.9
1,005	(965-1,060)	976	(930-1,020)	2.8*
225	(200-245)	201	(190-215)	10.6***
142	(130-160)	115	(105-125)	19.0***
3,480	(3,430-3,660)	3,330	(3,090-3,520)	4.3***
60	(58-62)	54.5	(51-57)	9.1***
21	(20-23)	20.5	(20-22)	2.3
178	(172-214)	178	(160-211)	0.0
131	(122-143)	119	(111-127)	9.1***
73	(69-81)	63	(57-67)	13.6***
21	(20-26)	18	(17-22)	14.2***
7.1	(7.0-7.30)	6.9	(6.80-7.10)	2.8***

Other Plasma Proteins, pH, and Lipids

In all plasmas examined, the levels of total protein, immunoglobulins, albumin, complement factors C3 and C4, α_1 -antitrypsin, haptoglobin, haemopexin, and caeruloplasmin were normal. The pH was close to 7.0 in all batches before as well as after treatment. The medians

before and after virus inactivation, percentages of decrease and significance levels are presented in table 3.

The mean decreases of triglycerides, cholesterol, and lipoprotein(a) were 50, 54 and 74%, respectively, as a result of the S/D step (data not shown). As already found when analysing the clotting factor and inhibitor levels, the lot-to-lot variation of all parameters was significantly lower in the pooled plasmas than in the single donor units.

Discussion

It is of great importance that a recent study proved that TNBP-plasma from homosexual individuals who were positive for HIV antibody did not transmit HIV or hepatitis viruses in chimpanzees [23]. More recently, it was demonstrated that lipid-enveloped viruses were actually inactivated in plasma by modified S/D procedures without dramatic reduction of the activities of labile proteins [13]. The authors could also show that S/D treated plasma was virtually free of neoantigens [Horowitz, personal commun.]. A further method to inactivate viruses in human plasma developed by others [24] using phenothiazine dyes in combination with visible light cannot be evaluated definitively, since decisive investigations such as studies concerning the capacity of hepatitis viruses inactivation, chimpanzee studies, and comprehensive in vitro characterization studies are still lacking.

The modified S/D treatment used in our study is superior to the formerly described S/D methods [13], since both TNBP and detergent are effectively reduced by the lipid extraction step to concentrations having no toxic effects even in massively or chronically transfused patients [25]. When analysing the results of our in vitro characterization of the S/D-treated plasmas the conditions of measurement have to be considered. The normal ranges of screening tests of blood coagulation, clotting factors, plasminogen, inhibitors, and other clinically important plasma proteins were established by analyzing plasma samples of healthy subjects after one freezing and thawing process. The same holds true for the 12 conventional single donor plasma units. However, the pooled plasmas were deep frozen and rethawed at least twice before analysis. In the lyophilized batches, the proteins were additionally stressed by the lyophilization step. Moreover, the S/D procedure is inevitably accompanied by an about 10% dilution. Thus, it is not surprising that most plasma protein levels drop to different degrees due to the virus inactivation process. On the other hand, it is remarkable that the activities or concentrations of most plasma proteins including the most

labile clotting factors VIII, V, and XI were still within the normal range after S/D treatment in all batches. APTT and TT values were also found to be normal in all lots, indicating that the treated plasmas contained no detectable heparin levels.

Unexpected findings are the marked decreases of protein S and α_2 -antiplasmin activities due to S/D treatment. The procedure also led to a marked drop of α_2 -antiplasmin antigen, whereas protein S concentration measured by enzyme immunoassay was not affected by virus inactivation. These results suggest that the low recovery of protein S activity was caused by actual inactivation of the protein. α_2 -Antiplasmin was obviously partly lost, probably during extraction of lipophilic substances. It will have to be determined whether α_2 -antiplasmin activity recovery can be improved by modifying the S/D process.

An additional advantage of the S/D-treated plasma over single donor plasma units is the fact that the interindividual variations of protein levels in the treated plasmas are extremely low as a consequence of pooling prior to virus inactivation. Thus, in contrast to conventional FFP the activities of the most important clotting factors and inhibitors can be declared and labeled by the manufacturer. This automatically results in improved therapeutic accuracy when using plasma for substitution of clotting factors or inhibitors.

TAT and FII splits are very sensitive markers of activation of hemostasis. The normal ranges published [26-28] can only be reproduced when analyzing plasma samples from healthy individuals gained under optimal conditions. So it is not surprising that two study groups found markedly different normal ranges of TAT [27, 28]. For this reason, we compared the TAT and FII splits levels measured in the pooled plasmas with the 'normal range' derived from 20 single donor FFP units, which were deep frozen and rethawed twice before analysis. Under these conditions we found elevated levels of TAT and FII splits in 2 and 1 deep frozen virus-inactivated plasmas, respectively. However, it has to be mentioned that these deep frozen batches treated with the S/D procedure were even frozen and thawed three times before measuring TAT and FII splits.

We found no increased thrombin-, factor Xa-, or kallikrein-like activities. These findings indicate that no complexes consisting of activated clotting factors and α_2 -macroglobulin were present in the treated plasmas, since activated clotting factors complexed by α_2 -macroglobulin retain their capacity to cleave chromogenic substrates while losing their ability to proteolyze their natural high-molecular-weight substrates [29]. Fibrin monomers and

D-dimers were within the normal range or not detectable in all batches. The low ratios of factor VIII one-stage and two-stage assay results are further proof that there are not any signs of activation of hemostasis due to the virus inactivation procedure [20-22].

In summary, the results obtained suggest that the compositions of the plasmas treated with the modified S/D process used in this study is very similar to that of single donor FFP. We cannot confirm the findings of others who found unacceptable clotting factor and heparin activities when characterizing similarly treated plasma pools in vitro [30, 31]. The clinical efficacy and safety of our S/D virus-inactivated plasma preparations have now to be confirmed in clinical studies.

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