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Peter Hellstern [a]
Hannelore Beeck [a]
Annette Fellhauer [a]
Antje Fischer [a]
Beate Faller-Stöckl
for the PCC Study Group [b]

[a] Institut für Transfusionsmedizin und Immunhämatologie,
[b] Section Hemostaseology of the Deutsche Gesellschaft für Transfusionsmedizin und Immunhämatologie (DGTI), Klinikum der Stadt Ludwigshafen, Deutschland

Factor VII and Activated-Factor-VII Content of Prothrombin Complex Concentrates[1]

Abstract

Background and objectives: The aim of this study was to determine the potencies of factor VII (FVII) and of activated FVII (FVIIa) in prothrombin complex concentrates (PCC). **Materials and methods:** We examined 56 lots of PCC from 5 manufacturers. Three brands were licensed preparations, and 1 product series had been involved in thromboembolic complications. FVII and FVIIa were measured using a two-stage amidolytic assay and a specific clotting assay, respectively. We also quantified FVII clotting activity by a one-stage assay reflecting a mixture of FVII zymogen and FVIIa. **Results:** All PCC contained substantial amounts of FVII, and FVIIa could be detected in all lots. There were marked differences between manufacturers and some significant variabilities between batches. The two lots involved in thromboembolic events contained considerably more FVIIa than the PCC still licensed. The lowest FVIIa potencies were observed in an experimental product series, indicating that PCC can be produced without activation of FVII during the manufacturing process. **Conclusion:** FVIIa is present in all PCC containing FVII. High FVIIa potencies may contribute to the thrombogenic potential of these preparations, and determination of FVIIa potencies should be included in the in vitro characterization of PCC.

Introduction

Since the composition of clinical prothrombin complex concentrates (PCC) varies according to the manufacturing method, those fractionators using strong ion exchangers commonly produce a 'four-factor' concentrate which includes factor VII (FVII), while those using weaker ion exchangers produce a 'three-factor' concentrate of FII, FIX and FX [1]. Three-factor PCC have been preferred to prevent and treat bleeding in hemophilia B [2]. PCC have been associated with thromboembolic complications including acute myocardial infarction [3–5] and disseminated intravascular coagulation (DIC) [6–8]. Thrombotic complications have continued to occur, after some manufacturing improvements and the addition of heparin to the preparations [4]. Thrombogenicity has mainly been attributed to activated clotting factors [9–13] or procoagulant phospholipid

[1] Dedicated to Prof. Dr. Hans-Erich Keller, Homburg-Saar

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E-Mail karger@karger.ch
Fax +41 61 306 12 34
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Prof. Dr. Peter Hellstern
Institut für Transfusionsmedizin und Immunhämatologie
Klinikum Ludwigshafen
Bremserstr. 79, D–67063 Ludwigshafen (Germany)
Tel. 621 / 5033500, Fax 621 / 5033520

[14]. Three-factor PCC are now gradually being replaced by high-purity FIX concentrates, which are obviously less thrombogenic [15–19].

Four-factor PCC are used for replacement of vitamin-K-dependent clotting factors and inhibitors protein C and protein S in phenprocoumon or warfarin overdose and liver disease [20–22]. These preparations are preferred to fresh frozen plasma in life-threatening situations since they reverse the respective coagulopathy more rapidly without causing volume overload [20–23]. Since the prescribed European Pharmacopoeia methods for assessing the thrombogenicity of PCC, nonactivated partial thromboplastin time (NAPTT) and thrombin fibrinogen clotting time (TFCT) are poor predictors of in vivo events in man [24], PCC continue to carry the risk of inducing thrombosis or DIC. Recently, we reported a PCC that had been involved in three fatal thromboembolic complications [25]. This preparation substantially differed from another licensed PCC due to its high activated FVII (FVIIa) content measured as proposed by Seligson et al. [13, 26].

FVIIa can now be quantified by a specific functional prothrombin time-based assay using a truncated form of human recombinant tissue factor and FVII-deficient plasma [27, 28]. This assay can be properly standardized using the 1st International Standard Factor VIIa Concentrate (89/688), as proposed by Hubbard and Barrowcliffe [29]. In contrast to FIIa, FIXa and FXa, which are rapidly removed from the circulation by antithrombin or antithrombin-heparin complexes [30], FVIIa has an in vivo half-life of about 2.5 h [13] since there is no naturally occurring fast-acting inhibitor of free FVIIa [31].

We focussed on measuring FVII and FVIIa in the presently commercially available four-factor PCC and PCC still under pharmaceutical development. We used a specific commercial clotting assay for the determination of FVIIa. FVII was quantified by a two-stage amidolytic assay (FVII:AM), utilizing the fact that FVII:AM represents functional FVII irrespective of its activity state, and by a one-stage clotting assay (FVII:C) which reflects a mixture of FVII zymogen and FVIIa [32].

Materials and Methods

Prothrombin Complex Preparations

Fifty-six batches of PCC from 5 manufacturers (German Red Cross Niedersachsen, German Red Cross Nordrhein-Westfalen/Serapharm, Centeon, Frankfurt, Germany; Immuno, Vienna, Austria; Octapharma, Ziegelbrücke, Switzerland; A–E) were partly purchased and partly provided by the manufacturers. All preparations were produced by batchwise adsorption-elution of cryosupernatant plasma with DEAE-Sepharose. The commercial preparations including the lots of manufacturer B contained between 0.4 and 5 IU heparin/10 IU of declared FIX. According to the specifications of the manufacturers, preparations C and E also contained between 0.08 and 0.6 IU antithrombin/10 IU declared FIX. Product series A1, A2 and A3 were provided by the same manufacturer according to different production protocols. The same holds true for product series B1 and B2 from manufacturer B and series D1 and D2 from manufacturer D. Four product series of PCC (manufacturer A: series A2 and A3; manufacturer D: series D1 and D2) were experimental preparations, and the remaining PCC were commercial preparations which had been put on the marked. All batches had passed the European Pharmacopoeia limits for NAPTT and TFCT. In 1994, the PCC of manufacturer B was taken from the market because of severe side effects. Two lots (B2) of this fractionator had been involved in thromboembolic complications and DIC [25]. Therefore, we shall subsequently describe this preparation B2 as a 'hot' PCC. These 2 lots and 2 lots from manufacturer C were more extensively analyzed. A standard preparation of an activated PCC (FEIBA®, 'Factor Eight Bypassing Activity') was kindly provided by Dr. Hartmut Lang, Immuno, Vienna, Austria, and served as control.

PCC were dissolved in twice distilled water to obtain a FIX concentration of 25 IU/ml as declared by the manufacturers. These stock solutions were analyzed within 3 h.

Determination of FVII and FVIIa

All parameters were determined in duplicate. FVII:C was measured with the semiautomated coagulometer KC10A (Amelung, Lemgo, Germany) using a one-stage clotting assay, human FVII-deficient plasma from Immuno, Heidelberg, Germany, and a rabbit thromboplastin (Neoplastin plus®; Boehringer, Mannheim, Germany). We diluted the PCC stock solutions 1:25, 1:50, 1:100 and 1:200 with FVII-deficient plasma to bring them into the measuring range. FVII:C was calculated from the results obtained in at least 3 PCC dilutions (mean values). Calibration was performed using a commercially available pooled plasma ('Reference Plasma 100%'; Immuno) which had been calibrated against the 1st International Plasma Standard for FVII (84/665). We quantified FVII:AM by a commercial two-stage chromogenic substrate assay (Immunochrom®; Immuno) using a COBAS Mira*plus* autoanalyzer from Roche, Grenzach, Germany. The test kit contained a secondary standard which had also been calibrated against the 1st International Plasma Standard for FVII (84/665).

We assayed FVIIa by a commercial test kit (Staclot FVIIa rTF; Stago, Asnières, France) using a coagulometer KC10A. This clotting assay is based on the selective FVIIa cofactor activity of a mutant tissue factor [27, 28]. A secondary standard derived from the 1st International Standard Factor VIIa Concentrate (89/688) served as a calibrator. PCC stock solutions were diluted using the dilution buffer of the test kit.

Intra-assay and interassay imprecisions were obtained by ten-fold or twenty-fold determinations of FVII and FVIIa activities in 'normal' and 'abnormal' control plasmas from Immuno and Stago, respectively.

In Vitro Characterization of the PCC B2 and C

The 2 lots which had been involved in thromboembolic complications [25] and 2 further lots from manufacturer C were more extensively analyzed.

FII and FX (FII:C, FX:C) were measured with the semiautomated coagulometer KC10A (Amelung) using one-stage clotting assays, human deficient plasmas from Immuno and a rabbit thromboplastin

Table 1. FVII:AM, FVIIa and FVII:C potencies as well as FVIIa/FVII:AM and FVII:C/FVII:AM ratios in the 56 lots of PCC from 5 manufacturers and in the activated PCC FEIBA

PCC	Batches n	FVII:AM/100 U FIX U		FVIIa/100 U FIX U		FVIIa/FVII:AM ratio		FVII:C/100 U FIX U		FVII:C/FVII:AM ratio	
		mean	min.–max.	mean	min.–max.	mean	min.–max.	mean	min.–max.	mean	min.–max.
A1	8	56	33–68	38	19–58	0.6	0.4–0.8	102	74–130	1.9	1.5–2.2
A2	13	33	23–66	32	19–56	1.0	0.6–1.7	53	37–92	1.6	1.2–2.3
A3	12	52	49–54	39	33–45	0.7	0.6–0.9	117	109–128	2.2	2.1–2.4
B1	3	62	51–73	54	40–64	0.8	0.7–0.9	140	108–170	2.2	2.1–2.3
B2	2	25	24–25	65	65–66	2.6	2.5–2.6	134	132–136	5.4	5.2–5.5
C	5	71	53–90	31	26–41	0.4	0.3–0.5	90	66–112	1.2	1.1–1.3
D1	4	29	24–37	71	52–109	2.4	2.1–2.9	121	90–169	4.1	3.7–4.4
D2	3	45	42–48	7	7–8	0.17	0.16–0.18	57	54–60	1.2	1.2–1.3
E	6	34	26–47	13	10–16	0.4	0.3–0.5	67	58–79	2.0	1.7–2.2
FEIBA	1					6.0				4.7	

min.–max. = Minimum–maximum.

(Neoplastin plus; Boehringer Mannheim). FIX (FIX:C) was quantified using the KC10A, human FIX-deficient plasma and phospholipid/kaolin from Immuno. We diluted the PCC stock solutions 1:25, 1:50, 1:100 and 1:200 with the buffer of the respective assay to bring them into the measuring range. By these predilutions and further dilutions in the respective assays, it was guaranteed that the heparin contained in the PCC did not influence the test results. FII, FIX and FX were calculated from the results obtained in at least 3 PCC dilutions (mean values). Calibration was performed using the 1st International Concentrate Standard for FII, FIX and FX (84/681).

All amidolytic assays were performed using a COBAS Mira*plus* autoanalyzer from Roche. FX (FX:AM) was quantified using an amidolytic assay from Haemochrom, Essen, Germany. FII:AM was determined using Ecarin from Pentapharm, Basel, Switzerland, and the chromogenic substrate Th_1 from Immuno. FXa-like activity was measured by a modification of the amidolytic assay for FX. Undiluted PCC was used as the sample, and the activator (Russel's viper venom + Ca^{2+}) was replaced by buffer.

We determined protein C and protein S activities by clotting assays from Boehringer. Antithrombin and heparin anti-Xa activities were quantified by chromogenic substrate assays from Boehringer and Haemochrom, respectively.

We measured thrombin generation after recalcification (TGt_{50}) according to Sas et al. [33] as modified by Cash et al. [24]. Human fibrinogen was purchased from Haemochrom. The NAPTT was assayed according to Kingdon et al. [9]. Platelet-poor plasma was from Immuno and phospholipid from Boehringer. The baseline NAPTT using platelet-poor plasma (Reference Plasma 100%; Immuno) and Tris-NaCl buffer was 190 s.

Statistical Analysis

The values are given as means and ranges (minimum–maximum) and as box plots. Pairwise comparisons were made using the Wilcoxon signed-rank test. Two-tailed probability values of below 0.05 were regarded as significant.

Results

FVII:C, FVII:AM and FVIIa Potencies

Reproducibility of FVII and FVIIa assays was good. Measurements of intra-assay and interassay imprecisions resulted in correlation coefficients below 10% for all assays and for all measuring ranges.

The potencies of FVII:AM, FVIIa and FVII:C as well as FVIIa/FVII:AM and FVII:C/FVII:AM ratios are given in table 1. It should be emphasized that the units of FVIIa are international units, measured with reference to the 1st International Standard Factor VIIa Concentrate (89/688) [29]. Thus, these units should not be mistaken for units of other coagulation factors, including FVII.

All PCC contained substantial amounts of FVIIa and FVII as determined by FVII:AM. However, there were marked differences between product series and partly marked batch-to-batch variations within product series. Altogether, FVII:AM varied from 23 to 90 IU/100 IU declared FIX and and FVIIa from 7 to 109 IU/100 IU FIX. In all lots, FVII:C potencies representing a mixture of native FVII and FVIIa were significantly higher than FVII:AM potencies, ranging from 37 to 170 IU/100 IU declared FIX.

The highest FVIIa potencies were observed in preparation B2, which had been involved in thromboembolic events and DIC [25], and in the experimental preparation D1 (fig. 1). FVIIa potencies of all batches of preparations C, D2 and E were clearly below the values measured in B2 and D1. However, the mean FVIIa potencies of the commercial product series A1 and C were only about 50% lower than the mean FVIIa potency of the hot PCC B2.

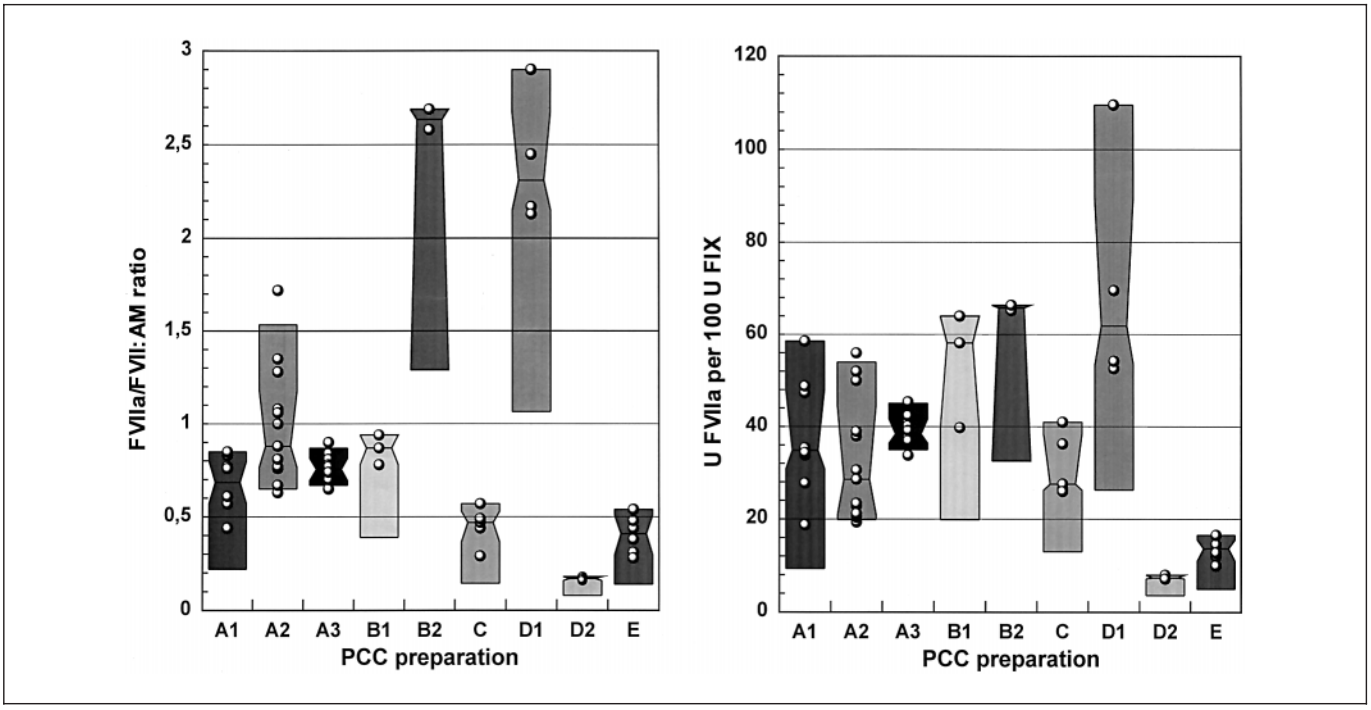

Fig. 1. FVIIa/FVII:AM and FVIIa potencies ratios in the 56 lots of PCC from 5 manufacturers. Mean values, single values and box plots. The bottoms of the boxes mark the 10th percentiles, the tops of the boxes mark the 90th percentiles, and the notches of the boxes mark the 25th and 75th percentiles, respectively. The horizontal lines separating the boxes into two parts mark the means.

The PCC could be subdivided into three categories according to their FVIIa/FVII:AM ratios: product series B2 and D1 with high mean ratios of 2.6 and 2.4, respectively; product series A1, A2, A3 and B1 with intermediate mean ratios ranging from 0.6 to 1.0, and product series C, D2 and E with low ratios ranging from 0.17 to 0.4 (fig. 1). We found a FVIIa/FVII:AM ratio of 6.0 in the activated PCC FEIBA, which served as a positive control. Considering that 1 IU of FVIIa measured with reference to the 1st International Standard corresponds to 22.4 ng FVII mass [Hubbard, A.R., NIBSC, pers. commun.] and that 1 IU FVII corresponds to 470 ng FVII mass [34], the percentages of FVIIa mass in relation to total FVII mass ranged from 1.4 to 3.8% in the commercial preparations A1, C and E and from 0.75 to 13.8% in the experimental PCC A2, A3, D1 and D2. Considering these data and FVII:AM potencies, all lots of the commercial preparations A1, C and E contained at least 25 IU of FVII zymogen/100 IU declared FIX. The two lots of PCC B2 involved in thromboembolic events contained 11.9 and 12.3% of FVIIa in relation to total FVII mass, respectively. In the FEIBA preparation, FVIIa mass represented 28.2% of the total FVII mass.

Table 2. In vitro characterization of the 2 lots of PCC B2 which had been involved in thromboembolic complications and of 2 lots from preparation C, which is still licensed. The FIX potencies as declared by the manufacturers B and C were 500 IU/vial and 600 IU/vial, respectively

Parameter	Preparation B2		Preparation C	
	lot 1	lot 2	lot 1	lot 2
FIX:C	490	535	980	845
FII:C	1,820	2,800	1,070	1,010
FII:AM	2,188	2,213	880	830
FVII:C	660	680	670	677
FVII:AM	125	125	490	540
FVII:C/FVII:AM ratio	5.3	5.6	1.4	1.3
FVIIa	325	330	220	160
FX:C	885	935	735	715
FX:AM	910	1,360	595	610
Protein C	630	590	495	470
Protein S	n.d.	n.d.	260	270
Antithrombin	n.d.	n.d.	20	20
Heparin	335	405	285	285
FXa-like activity	n.d.	n.d.	n.d.	n.d.

Values are given in IU/vial. n.d. = Not detectable.

FVII:C/FVII:AM ratios as a measure of FVIIa were also substantially greater in PCC B2 and D1 than in the remaining preparations. The mean FVII:C/FVII:AM ratio of PCC B2 was even higher than the value for the FEIBA control.

In vitro Characterization of the PCC B2 and C

The results are given in table 2. TGt_{50} exceeded 60 min in all 4 PCC. NAPTT was not shortened by any of the 4 preparations up to a dilution of 1:2,048. There were striking differences between preparations B2 and C. The 2 lots of PCC B2 were overloaded with FII and did not contain antithrombin and protein S activities. FVIIa potencies and FVII:C/FVII:AM ratios of preparation B2 were substantially greater than in preparation C.

Discussion

The reason for our in vitro investigations were two fatal cases of thromboembolism and one fatal case of multiorgan failure as a result of DIC occurring immediately after administration of PCC [25]. The same preparation had already caused life-threatening multiorgan failure in a further case [8]. This four-factor PCC differed strikingly from another commercial preparation due to its high FVIIa content (table 2).

We analyzed all brands of four-factor PCC which had been put on the market during the last years. We also included 4 experimental product series in our investigations. All batches had passed the European Pharmacopoeia limits for NAPTT and TFCT.

FVIIa could be detected in all batches. This is not surprising since FVIIa represents approximately 1% of total FVII mass circulating in the plasma of normal individuals [27, 28, 35]. However, there were considerable differences between product series and partly significant variabilities between batches within product series (fig. 1). The same holds true for FVIIa/FVII:AM ratios.

The amount of FVIIa present in the lots of product series B2, which had been involved in thromboembolism, represented approximately 12% of total FVII mass. This is about 4–6 times higher than the values found in the PCC A1, C and E which are still licensed. These findings indicate that the manufacturing processes of PCC can obviously induce the formation of FVIIa. On the other hand, the very low FVIIa potencies and FVIIa/FVII:AM ratios of the experimental preparation D2 demonstrate that PCC can be produced without any induction of FVII activation.

FVII:AM potencies also markedly differed from product series to product series. All licensed preparations contained at least 25 IU of FVII zymogen/100 IU of FIX. Unfortunately, there are no reliable data establishing the minimum FVII levels required for hemostasis in different clinical situations [36]. However, the question should be posed whether FVII potencies as high as 90 IU/100 IU FIX are necessary for the treatment of acquired deficiencies of vitamin-K-dependent clotting factors. Though high FVII:AM potencies must not necessarily lead to high FVIIa potencies, the results obtained when analyzing the PCC of manufacturer C demonstrate that this is a potential risk. Despite low FVIIa/FVII:AM ratios, mean FVIIa potency was only about 50% lower than in the hot preparation B2.

As expected, FVII:C potencies representing a mixture of FVII zymogen and FVIIa were significantly higher than FVII:AM potencies. These findings suggest that FVII:C is not suitable for establishing potencies of native FVII in PCC.

The correlation between high FVIIa potencies in PCC and their thrombogenic potential remains to be determined. The thrombogenicity of PCC has been the subject of numerous in vitro and in vivo studies in animal models. Thrombogenicity has mostly been ascribed to FIXa [10–12] and FXa [10]. Recently, Gray et al. [37] found a significant positive correlation between FIXa levels measured by a sensitive chromogenic assay and in vivo thrombogenicity of high-purity FIX concentrates when unphysiologically high doses of 200 IU/kg were administered to rabbits. However, the FIXa levels found were very low and could be completely neutralized by adding heparin and antithrombin. Previous work has already demonstrated that the addition of heparin and antithrombin to PCC or the prophylactic administration of heparin resulted in a marked reduction of FIXa, FXa and thrombogenicity since FIXa and FXa are rapidly removed from the circulation by antithrombin or antithrombin-heparin complexes [10, 30]. However, thrombotic complications have continued to occur after addition of heparin to PCC previously recommended by the FIX Task Force of the International Society on Thrombosis and Hemostasis [4]. A more recent study showed that infusion of 50 IU/kg of PCC containing antithrombin, but no heparin, to hemophilia B patients resulted in significant increases in thrombin-antithrombin complexes, prothrombin activation peptide fragment F1+2 and FX activation peptide, but not FVIIa [38]. No increases in the markers of activated coagulation were observed when a high-purity FIX concentrate was administered. The authors concluded that activation of coagulation in vivo must be linked to the presence of FIXa. Our in vitro studies have demonstrated that the 2 PCC involved in thromboembolism or DIC were virtually free of FXa. Unfortunately, we had no assay available for quantifying FIXa.

Finally, it remains uncertain whether FIXa and FXa are mainly responsible for the thrombogenicity of PCC.

Seligson et al. [13] determined FVII and FVIIa in 17 lots of PCC from 4 manufacturers and in 8 lots of 2 brands of activated PCC. FVIIa was estimated using the FVII:C/FVII:AM ratio [26]. FVIIa was present in all preparations, and higher FVII:C/FVII:AM ratios were found in the 2 brands of activated PCC. Infusion studies in man revealed a mean intravascular half-disappearance time of 144 min for FVIIa. These findings are in accordance with the fact that there is no naturally occurring fast-acting inhibitor of free FVIIa [31]. As free FVIIa is not rapidly neutralized by antithrombin-heparin complexes, it is conceivable that high FVIIa potencies contribute to the thrombogenic potential of PCC. This is supported by our findings that the PCC involved in thromboembolic events and the activated preparation FEIBA contained substantially higher FVIIa potencies than the licensed PCC included in our in vitro investigations. On the other hand, very high doses of recombinant FVIIa are used for treatment or prevention of bleeding in patients with FVIII and FIX inhibitors without causing thromboembolism [39, 40].

The fatal events after administration of PCC B2 have shown that thromboembolism or DIC occur as a consequence of several factors, including predisposing factors such as a hypercoagulable state or thrombophilia, drug interactions between PCC, aprotinin and protamin [25] and the composition of PCC. The PCC in question contained not only high FVIIa potencies, but was also overloaded with FII, while being virtually free of antithrombin and protein S activity (table 2). Our results concerning NAPTT and TGt_{50} confirm former statements that these assays have a low predictive value for assessing the thrombogenic potential of PCC.

Our findings suggest that the determination of FVIIa using a direct assay standardized against the 1st International Standard Factor VIIa Concentrate should be included in the measures for characterizing PCC. FVII potencies have to be quantified by a two-stage amidolytic assay, since FVII:C assessed by a one-stage clotting assay may lead to an overestimation of FVII due to the presence of FVIIa. The importance of high FVIIa potencies in PCC for their in vivo thrombogenicity has to be determined by in vivo studies.

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Announcement

International Society of Blood Transfusion Jean Julliard Prize

Applications are invited for the 16th Jean Julliard Prize, which was established by the International Society of Blood Transfusion in memory of its first Secretary-General. The Prize will be awarded during the 25th International Congress in Oslo, Norway, June 27–July 2, 1998.

Entrance is reserved for scientists under 40 years of age, in recognition of recently completed work on blood transfusion or related subjects.

In order to qualify, candidates should forward six copies of their submission, in English, including a curriculum vitae, to the Secretary before January 31, 1998.

The prize of 3,000 CHF will be awarded during the Congress. The successful candidate will be required to give a presentation on his/her submission.

Full regulations are available on request from the Secretary-General, National Blood Service/Lancaster, PO Box 111, Royal Lancaster Infirmary, Ashton Road, Lancaster LA1 4GT, UK.