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Chromatographic purification and properties of a therapeutic human protein C concentrate

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Abstract

Protein C deficiency (inherited and acquired) has a relatively high incidence rate in the general population worldwide. For many years, protein C deficient patients have been treated with fresh frozen plasma, prothrombin complex concentrates, heparin or oral anticoagulants, which all have clinical drawbacks. We report the production process of a highly purified human protein C concentrate from 1500 l of cryo-poor plasma by a four-step chromatographic procedure. After DEAE-Sephadex adsorption, protein C was separated from clotting factors II, VII and IX by DEAE-Sepharose FF and further purified, using a new strategy, by an on-line chromatographic system combining DMAE-Fractogel and heparin-Sepharose CL-6B. In addition, the product was treated against viral risks by solvent-detergent and nanofiltration on 15-nm membranes. The protein C concentrate was essentially free of other vitamin K-dependent proteins. Proteolytic activity was undetectable. Neither activated protein C, prekallikrein activator, nor activated vitamin K-dependent clotting factors were found resulting in good stability of the protein C activity. In vitro and in vivo animal tests did not reveal any sign of potential thrombogenicity. The final freeze-dried product had a mean protein C concentration of 58 IU/ml and a mean specific activity of 215 IU/mg protein, corresponding to over 12 000-fold purification from plasma. Therefore, this concentrate appears to be of potential benefit for the treatment of protein C deficiency.

Keywords: Protein C; Proteins; Factor II; Factor VII; Factor IX

1. Introduction

The protein C pathway appears to be involved in both anticoagulant and anti-inflammatory activities. It displays anticoagulant activity by inhibiting factor Vc and factor VIIIc activities [1] and, probably, by activating plasma protein C through activated protein C, as shown recently [2]. Its anti-inflammatory properties are exerted through protein S and the C4bBP, a regulatory protein of the complement system and by avoiding the synthesis of tumor necrosis factor [3]. Also, its in vivo profibrinolytic properties have been described [4].

The most common treatment of protein C deficient patients is still fresh frozen plasma (FFP), in spite of low protein C content (1 IU/ml or lower) and specific activity (0.017 IU/mg protein), thus requiring massive fluid administration and exposing the

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patient to some viral risks. A readily available enriched protein C preparation is the prothrombin complex concentrate (PCC) but it is poorly purified as indicated by its low specific activity of 1 IU protein C/mg protein. Efforts to purify and concentrate protein C started in the 1970s. Several laboratory methods based on barium salt or ammonium sulfate precipitation, ion exchange on Sephadex, and affinity chromatography were developed for human plasma protein C as reported by Kisiel [5]. Later, immunoaffinity protein C preparations or pilot-scale fractions were described [6–9] and one of these preparations has been clinically evaluated [10–12]. Recombinant technology has been recently applied to produce an activated protein C concentrate for the treatment of severe sepsis [13].

Since the immunopurified and recombinant protein C or activated protein C may not always be easily available [14–16], alternative production methods of plasma-derived protein C need to be developed. We describe here an original large-scale production process for a highly purified plasma derived protein C, based on a four-step adsorption chromatography. The process is compatible with factor IX and other therapeutic plasma proteins production. This protein C concentrate is non-thrombogenic and exhibits good stability without the need for a protein stabilizer such as albumin. The product meets currently required viral safety profiles thanks to the combination of two distinct specific viral reduction steps (solvent-detergent (SD) and nanofiltration) targeting lipid enveloped and non-lipid enveloped viruses.

2. Materials and methods

2.1. Materials

Plasma was collected in France and complied with the specifications of plasma for fractionation. It was obtained by centrifugation of whole blood within 6 h of the collection process.

The following chromatographic gels were used: DEAE-Sephadex A-50, DEAE-Sepharose FF and heparin-Sepharose CL6B from Pharmacia, and DMAE-Fractogel EMD from Merck.

2.2. Production process

Protein C production process was developed as a by-product of the factor IX purification scheme [17].

2.2.1. Pre-purification

Each batch of protein C concentrate was prepared from a protein C-containing fraction of the factor IX purification process starting from 1500 l of cryo-poor plasma which were adsorbed on DEAE-Sephadex A50, as described previously [18].

2.2.2. Solvent-detergent treatment

The DEAE-Sephadex eluate containing the vitamin K dependent proteins was virally inactivated by SD using a combination of tri-n-butyl phosphate (TnBP) (0.3%) and Tween-80 (1%) at 25±1 °C for at least 6 h [19]. The virus inactivating agents were subsequently removed by chromatography on DEAE-Sepharose FF that also contributed to the purification of protein C [20]. The protein C pre-purified fraction was eluted by a buffer containing 0.02 *M* trisodium citrate (TSC), 3 g/l lysine, and 0.28 *M* NaCl. Dialysis and concentration of the eluted protein C fraction were performed with the same elution buffer, with the exception of the NaCl content (0.10 *M*; buffer A) using a Pall-Filtron ultrafiltration system (M_r 10 000 cassettes).

2.2.3. Polishing chromatographic purification

A 4-l column (diameter 11.3 cm) packed with DMAE-Fractogel EMD was mounted and connected in series with a 1-l column (diameter 11.3 cm) packed with heparin-Sepharose CL6B. The concentrated protein C fraction was first loaded onto the DMAE-Fractogel resin, which was washed by buffer B prior to protein C elution. Columns are then connected, as described, to elute protein C which was directly injected into the second column. Columns were then disconnected to elute the purified protein C from the immobilized heparin gel.

The DMAE-Fractogel column was equilibrated with buffer A at a flow-rate of 10 l/h. After protein loading, the gel was washed with buffer B (0.02 *M* TSC, 9.1 g/l lysine, 0.15 *M* NaCl, pH 6) to elute inter-α-trypsin inhibitor (ITI). Then, buffer C (0.02 *M* TSC, 3 g/l lysine, 0.10 *M* NaCl, pH 5) was

applied to re-equilibrate the mobile phase by lowering the pH. The heparin-Sepharose CL6B column was equilibrated with buffer D (0.02 *M* TSC, 3 g/l lysine, 0.15 *M* NaCl, pH 5.0) before connecting both columns in series. Buffer D allowed protein C elution from DMAE-Fractogel (at a flow rate of 4.0 l/h) and its direct subsequent adsorption onto heparin-Sepharose CL6B gel. Columns were then disconnected and protein C was eluted from the latter by lowering the ionic strength and increasing the pH with buffer E (0.02 *M* TSC, 3 g/l lysine, 0.10 *M* NaCl, pH 7.2) at a flow rate of 8 l/h. The eluate was then concentrated to ~55 IU protein C/ml with buffer E using M_r 10 000 cassette membranes (Pall-Filtron).

2.2.4. Nanofiltration

The final product was formulated with 0.02 *M* TSC, 3 g/l lysine and 0.1 *M* NaCl, pH 7.2. The ionic strength and pH of the protein solution was in the physiological level (350±50 mosmol/l and 7.2±0.2, respectively). Nanofiltration was achieved through one single filter with a mean pore size of 15 nm (Planova 15 N) from Asahi (Japan) using a ''dead-end'' filtration system. The surface area of the filter was 0.3 m^2 and the working pressure was less than 0.6 bar. The whole nanofiltration procedure was completed in less than 2 h. The nanofiltered protein C solution was then sterile-filtered on a 0.22-μm filter (Pall), dispensed into vials containing 10 ml of protein solution and freeze-dried.

2.3. Analytical methods

Protein content was determined by the Bradford method using Coomassie blue G250 as the staining agent (reagent 23200, Pierce) and albumin as a standard. Amidasic activity of protein C was measured using a synthetic chromogenic substrate: CBS42.46 (reagent Stachrom Protein C, Stago). Assessment of functional protein C and activated protein C were both based on the prolongation of the activated thromboplastin time and were performed using the Staclot Protein C test (Stago). The test for activated protein C was carried out in the absence of ''Protac'' (kit reagent for activating protein C). The activity assays for factor II, VII, IX and X were carried out as described previously [18]. Protein S:Ag was measured by enzyme-linked immunosorbent assay (ELISA; Stago).

Proteolytic activity of the protein C concentrate was evaluated using the chromogenic substrate S2288 (Chromogenix), prekallikrein activator (PKA) by chromogenic substrate S2302 (Chromogenix), and NAPTT (non-activated partial thromboplastin time) test and FCT (fibrinogen clotting time) as reported before [20]. Sodium dodecylsulfate–polyacrylamide gel electrophoretic (SDS–PAGE) analyses were performed using 4–15% polyacrylamide gradient gels on a PhastSystem (Pharmacia). Electrophoretic bands were detected by silver staining. Immunoblots were prepared with antibodies specific for purified protein C and possible expected contaminants. TnBP and Tween 80 levels were determined using methods developed at the New York Blood Center [18].

2.4. Animal studies

In vivo thrombogenicity was determined using a modified Wessler test [20] while the presence of vasoactive substances in protein C batches was monitored using a rat model [21]. Acute toxicity was tested intravenously in mice at 25 ml/kg [21].

2.5. Stability studies

Stability studies were carried out on the final product after reconstitution of the freeze-dried material and incubation of the protein solution for up to 24 h at room temperature. Samples were tested for protein C activity after 4- and 24-h incubation in the liquid state and compared to the activity found immediately after reconstitution.

3. Results

3.1. Protein C purification

The optimized method developed for protein C purification and the whole procedure are briefly summarized here. Plasma was subjected to cryoprecipitation and the resulting supernatant was adsorbed on DEAE-Sephadex A-50, subsequently sub-

jected to a specific viral inactivation treatment by SD and chromatographed on DEAE-Sepharose FF. The protein C pre-purified eluate was then run onto a DMAE-Fractogel EMD chromatographic column connected in series with a heparin-Sepharose column. The purified protein C eluate was concentrated, subjected to a viral removal step by nanofiltration on a 15-nm membrane, sterile filtered and filled aseptically into vials. The final product was then freeze-dried.

Yield and purity of protein C achieved after each chromatographic step in the protein C production process were calculated from 1500 l of human plasma (Table 1). Forty-five percent of the protein C activity was recovered in the DEAE-Sephadex eluate together with factors II, IX and X while the bulk of albumin and immunoglobulins and other plasma proteins eluted in the breakthrough. These contaminating clotting factors were present in amounts similar to that of protein C. Factors II and IX were then separated by chromatography on the DEAE-Sepharose FF column (factor II/protein C and factor IX/protein C ratios were <1 and $<2.5\%$, respectively) but the protein C fraction was still heavily contaminated by factor X (factor X/protein C ratio 54%). The protein C recovery was 30% after DEAE-Sepharose FF.

The elution profile obtained on the DMAE-Fractogel/heparin-Sepharose system is shown in Fig. 1. Almost half of the protein load was found in the DMAE breakthrough fraction (F1) with little loss of protein C activity ($<1.5\%$). The wash fraction (F2) contained other contaminant proteins, mainly ITI. Purified protein C was recovered in the heparin eluate (F4) with a specific activity of >200 IU/mg protein. Most factor X was strongly adsorbed on the DMAE-Fractogel resin and was desorbed by the 2 *M* NaCl-containing buffer used for column regeneration (F6).

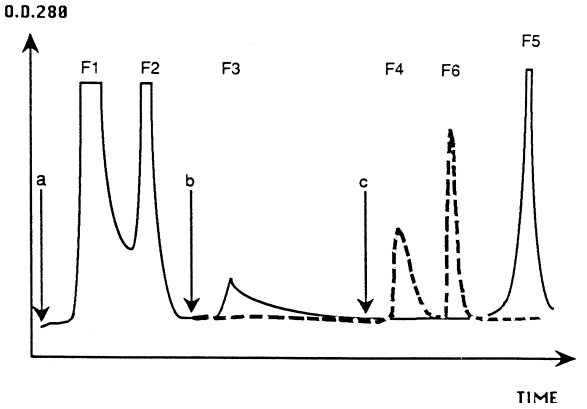

Fig. 1. Chromatographic profile of protein C purification on DMAE-Fractogel EMD (continuous line) and heparin-Sepharose (dotted line) columns connected in series. F1: DMAE filtrate, F2: DMAE wash, F3: heparin filtrate, F4: heparin eluate, F5: DMAE regeneration, F6: heparin regeneration. (a) Loading on DMAE-Fractogel column; (b) elution on DMAE gel/loading on heparin gel; (c) elution from heparin gel.

3.2. Biochemical properties of the protein C concentrate

The main biochemical characteristics of the protein C concentrate were evaluated for six consecutive industrial batches and are cited in Table 2. The final product had an average potency of 58 IU/ml (anticoagulant activity) and 61 IU/ml (amidasic activity). Total protein was 0.27 g/l and the mean specific activity was 215 IU protein C/mg protein. The

Table 1
Purification of protein C by ion-exchange chromatography

Purification parameter	DEAE-Sephadex A50	DEAE-Sepharose FF	DMAE-Fractogel/ heparin-Sepharose
Factor IIc/protein C[a]	106%	<1%	<0.1%
Factor IXc/protein C[a]	95%	2.1%	<0.2%
Factor Xc/protein C[a]	96%	54%	1.9%
Yield/step	45%	30%	73.5%
Specific activity (SA) (IU/mg protein)	1.5	2.0	215
Purification factor[b]	88	118	12 647

[a] Activity ratio.
[b] From plasma.

Table 2
Characteristics of the protein C concentrate (mean, $n=6$)

Protein content (g/l)	0.27±0.11
Protein C activity (anticoagulant) (IU/ml)	58±18
Protein C activity (amidolytic) (IU/ml)	61±20
Specific activity (IU protein C/mg protein)	215±40
Factor IIc (IU/ml)	<0.10
Factor VIIc (IU/ml)	<0.15
Factor IXc (IU/ml)	<0.10
Factor Xc (IU/ml)	1.1±0.8
Protein S:Ag (IU/ml)	<0.01
S2288 (Δ AU/min)	<0.005
Activated protein C (EIU/ml)	<0.01
PKA (IU/ml)	<0.02
NAPTT 1/10 (s)	250±15
NAPTT 1/100 (s)	227±10
FCT at 20 °C (h)	>24
FCT at 37 °C (h)	>6
Wessler (ED50) (IU protein C/kg)	>766

concentrate was essentially free of other vitamin K-dependent proteins such as factors II, IX and VII, and protein S which were below detection levels. Only residual amounts of factor X were found (1.1 IU/ml factor Xc corresponding to a factor Xc/protein C ratio <2%). Average pH and osmolarity were 7.2 and 350 mosmol/l, respectively. The high purity of the concentrate was confirmed by SDS–PAGE analysis, which only revealed a doublet band (Fig. 2A), identified as protein C by immunoblotting (Fig. 2B). The components of the doublet band are likely to be the α-protein C and β-protein C chains, respectively [22].

Proteolytic activity was practically nil using the chromogenic substrate S2288 (<0.005 Δ AU/min). PKA content was below detectable levels (<0.02 U/ml). No protein C activation was detected (activated protein C<0.01 U/ml). SDS–PAGE and immunoblotting of the purified protein C did not evidence any band with molecular mass lower or higher than those corresponding to protein C, indicating that the purification process did not alter the native protein (Fig. 2).

3.3. Thrombogenicity studies

Table 2 shows the results of in vitro and in vivo thrombogenicity tests. Average NAPTT values were 250 and 227 s for protein C samples diluted to 1/10 and 1/100, respectively (buffer was at 227 s). The corresponding mean in NAPTR (non-activated partial thromboplastin ratio) was 1.1 and 1, respectively. FCT performed at 20 and 37 °C were >24 and >6 h for all tested samples, respectively.

3.4. Animal studies

Intravenous administration of the protein C concentrate in rabbits using a vein stasis model did not induce any blood clot formation even at high doses

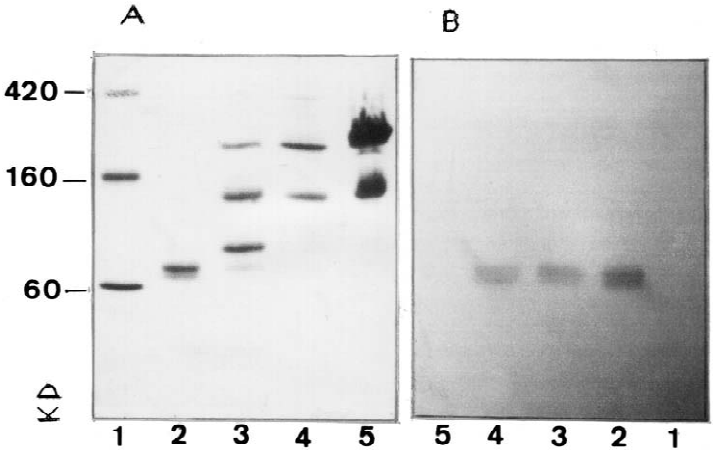

Fig. 2. SDS–PAGE (A) and immunoblotting (B) of protein C purification fractions. Lanes: 1=protein standards; 2=heparin eluate; 3=DMAE regeneration; 4=heparin regeneration; 5=DMAE filtrate+wash fraction. Protein standards: fibronectin, immunoglobulin G and albumin (laboratory purified human proteins).

(Wessler DE50>766 IU protein C/kg). Similarly, no sign of intolerance was observed in the rodent model. In the rat model, heart rate and arterial pressure of animals did not significantly change after injection of protein C concentrate at a dose of 50 IU protein C/kg body mass. Also, the acute toxicity test performed in mice using a single intravenous dose of 25 IU/kg body mass did not cause lethality nor signs of toxicity after 7 days of follow-up.

3.5. Viral validation studies

Preliminary virus inactivation studies of the SD treatment have been made using the model viruses, vesicular stomatitis virus (VSV) and Sindbis virus. Both lipid-enveloped viruses were inactivated within 2 h of treatment. The total kill was >5.6 and >4.8 $\log_{10}$ for VSV and Sindbis virus, respectively [20]. The viral inactivation agents, TnBP and Tween 80, were removed by the chromatographic wash on the DEAE-Sepharose gel and were undetectable in the final product (<1 and <10 ppm, respectively).

3.6. Stability studies

The stability of the reconstituted protein C concentrate was excellent, as demonstrated by the protein C recovery of 98.4 and 98.2% after 4 and 24 h of incubation, respectively. The stability of this preparation may be linked to the absence of proteases. This also avoided the stabilization by human albumin as done in some other plasma protein preparations.

4. Discussion

Thrombophilia may represent a major clinical complication in patients with certain platelet defects, hyperlipidaemia and protein C pathway defects [23]. Protein C deficiency may be congenital or acquired. The congenital defect is due to either a homozygous genotype, associated with purpura fulminans neonatalis, often leading to lethal thromboembolic disease in early infancy, or a heterozygous condition that is a recognized pathology that may result in recurrent thromboembolic complications usually in adults [24,25]. Acquired protein C deficiency has been reported to be triggered by severe bacterial sepsis (e.g. meningococcal septicaemia), chronic and acute liver disease, disseminated intravascular coagulation (DIC), malignancy, post-surgical hypercoagulable state, etc. [26].

Standard clinical management of heterozygous protein C deficiency is still based on oral anticoagulation or heparin therapy while the homozygous protein C deficient patients are given FFP, protein C concentrate or coumarin derivatives [24]. However, in some cases, heparin and coumarin were found to lack good clinical tolerance or to be ineffective in the control of thrombotic tissue necrosis while the replacement therapy with a protein C concentrate restored the microcirculation in those patients [25,27–30]. For a long time, hereditary protein C deficient patients have been known to respond well to protein C replacement therapy [28,30–33]. However, it is only recently, although Marlar had already suggested in 1985 [34] the importance of protein C substitution therapy in acquired protein C deficiency, that protein C replacement has been applied and shown to be effective to treat acquired protein C deficiency especially in sepsis-associated purpura fulminans [14,16,35,36].

Obviously, the lack of protein C in its purified and concentrated form until recently did not permit demonstration of the clinical interest of protein C in cases of acquired protein C deficiency. Protein C concentrates have been shown to be particularly effective when administered at a relatively early stage of the disease or before the onset of multiple organ failure [37]. Also, there is some evidence that protein C concentrates may be beneficial in DIC cases for at least restoring the normal anticoagulant pathway [38,39]. However, in spite of its current recognized clinical efficacy, the availability of purified protein C concentrates remains still limited worldwide resulting in a continuous dependence on FFP or other less efficient therapies. By nature, plasma administration will induce a protein and fluid overload in the patient's blood stream and the physiologic response to normalize the coagulation parameters may be performed at a slower rate than with a protein C concentrate. Moreover, frequent product administration is required to treat protein C deficiency due to the relatively short half-life of the protein resulting in an uncomfortable therapy for the patient. The first protein C concentrates available

were the prothrombin complex concentrates but, as many practitioners have pointed out, such poorly-purified products may induce a significant risk of thromboembolic complications, such as venous thromboembolism, myocardial infarction and DIC [40]. These thrombotic complications whose risk increases when large and repeated doses are administered to patients with other risk factors such as surgery and trauma, can be attributed to residual impurities and/or activated factors in PCC preparations [41]. Meanwhile, some highly purified factor IX concentrates have been developed for the substitution of PCC in the treatment of hemophilia B patients [18,20,42]. The fact that these purified products reduce the risk of thromboembolic complications [43] supports the interest for other preparations such as protein C, protein S, factor VII and factor X in a highly purified and concentrated form to ensure safer replacement therapy for deficient patients.

For several decades, the industrial production of highly purified protein C has been hampered due to its very low concentration in human plasma (~4 mg/l plasma) and to the technical difficulties, using conventional chromatographic means, of isolating it from other vitamin K-dependent proteins that have similar physico-chemical properties. In consequence, purified and concentrated human protein C was only available in its immunopurified version until recently. The first report on protein C isolation using monoclonal antibodies seems to be that of Esmon et al. [9]. Based on this method, protein C and activated protein C preparations were purified and administered to healthy humans in Japan in 1990 to follow their effect on coagulation and fibrinolysis [4]. Some years later, an immunopurified plasma-derived protein C preparation was developed in Europe. This protein C concentrate which is vapor-heated and stabilized with human albumin presents a specific activity of 14 IU protein C/mg protein [7].

From the late 1980s, the advent of more performing chromatographic resins with higher specificity and selectivity allowed the recovery of proteins present in trace quantities in human plasma. High quality products with high yields were then obtained for the first time at the industrial level for factor VIII [44], von Willebrand factor [45], factor XI [46] and factor IX [47] among others. Following the same perspective, the development of a plasma protein C concentrate benefited from these advanced chromatographic technologies. The present protein C process, based on ion-exchange/affinity chromatographic techniques, has the major advantage of being compatible with the production of a highly purified factor IX concentrate while providing reasonable yields for protein C activity after DEAE-Sephadex and DEAE-Sepharose. The industrial protein C purification was designed to provide a stable and a highly purified protein C preparation. This was accomplished by connecting the chromatographic columns in series to allow rapid protein C binding to the heparin column after elution from the DMAE-Fractogel resin. Consequently, the contact time of free protein C in the acidic buffer solution was greatly reduced, thus preserving its biological activity, reducing the risk of microbial contamination and decreasing the overall processing time. Yields were consistently higher than 70% for the DMAE-Fractogel EMD/heparin-Sepharose system. The excellent stability of the final product complied with the absence of protein degradation and/or activation, lack of proteolytic activity, and, particularly of activated clotting factors. These properties are considered to be very relevant as such proteases have been implicated in the clinical thrombogenicity of PCC [48].

The infusion of supranormal levels of clotting factors and, possibly, activated factors, in PCC preparations is thought to be one of the major risks of thrombotic complications in hemophilia B patients [49]. The absence of thrombogenic potential associated with the purified protein C concentrate has been demonstrated by in vitro and in vivo tests using a high dose of the concentrated protein C. As thrombogenicity has been associated with low purity products, lack of thrombogenic events is consistent with the high purity level of this concentrate and, particularly with the absence of proteases. Thus, the purified protein C concentrate appears electrophoretically homogeneous without indication of protein C denaturation (protein C aggregates and/or protein C degradation products) as shown by immunoblotting.

This protein C concentrate has a potentially high degree of viral safety as the process includes dual viral reduction treatments based on two different principles. The first is an SD incubation that is well

recognized for its capacity to inactivate the more pathogenic lipid enveloped viruses [50]. The protein C concentrate is obtained from the same SD treated fraction as that used to produce a high-purity factor IX preparation [20] which has been used in clinics since 1989 and has been shown not to transmit human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV) [51]. The second treatment, nanofiltration, is based on the retention of viruses using filter membranes with porosities in the order of a few nanometers. This method has already demonstrated its potential in factor IX and factor XI industrial processes to eliminate or reduce viruses as small as 20 nm in diameter [52] such as the parvoviruses and has, now, also been adopted for other plasma protein concentrates by several manufacturers [53]. This step is aiming more particularly at the removal of non-enveloped blood borne viruses like parvovirus B19 and hepatitis A virus, while also providing an added safety margin for other viruses and potentially resistant infectious agents that could contaminate human plasma in the future, including prions [54].

The interest of this protein C purification strategy relies on the use of mild procedures for both chromatographic, which does not involve the use of chaotropic eluents, and viral reduction methods (gentle chemical and filtration procedures based on regenerated cellulose membranes) which diminish the risk of protein alteration and protects the biological activity of protein C. Although oxidation and nitration may be induced during isolation and purification procedures causing an in vivo immune response, under the conditions used for the described protein C purification, it is much less likely that these events happen with plasma derived proteins than with their recombinant counterparts [55]. This is supported by the biochemical data and the specific activity, which is close to that of the pure protein showing no alteration of the final protein C product, and the clinical studies which evidenced no side-effects, including inhibitor formation [56]. The present method also prevents the risk of contamination with murine proteins that may leach from immunoaffinity purification systems [57]. In addition, our process provides a stable protein C preparation, which avoids the need for a protein stabilizer, thanks to simple elimination of proteolytic activity during the heparin-Sepharose chromatography. The resulting product is a stable and highly purified concentrate with an SA of 215 IU protein C/mg corresponding to a purification factor of over 12 000 from the starting plasma.

Recently, an activated protein C produced by recombinant technology has been proposed for treating septicaemia; clinical trials demonstrated a lower mortality rate, although with an increased risk of bleeding as compared to standard therapies [58,59]. This high-tech biopharmaceutical product, which may also be used in cases of sepsis-induced protein C deficiency, is likely to be more costly than a plasma-derived product. In conclusion, a cost-effective high-quality protein C concentrate can be obtained from human plasma using a chromatographic process compatible with current plasma fractionation scheme. Clinical studies have demonstrated the in vivo efficacy, viral safety and good tolerance of this protein C concentrate [56].

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