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A Therapeutic Concentrate of Coagulation Factors II, IX and X from Citrated, Factor VIII-Depleted Plasma

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Abstract. A simple procedure is described for the large-scale absorption on to DEAE-cellulose of coagulation factors II, IX and X from citrated, factor VIII-depleted plasma. The coagulation factors are eluted frontally from the exchanger in a high yield and in a form suitable for therapeutic use, without further fractionation. The lyophilised concentrate is very stable without the addition of heparin and, when redissolved to iso-osmolar solution, contains approximately 30 U/ml factors II, IX and X, 250–300 times purified from the starting plasma. The effectiveness of the concentrate in the treatment of haemophilia B is discussed.

Introduction

Until 1967, only fresh-frozen plasma could be offered in Scotland for correcting deficiencies of coagulation factors II, VII, IX and X. Such treatment was frequently inadequate to maintain haemostatic levels of factor IX in patients with severe haemophilia B during major bleeding episodes or surgery.

Since 1967, the Protein Fractionation Centre has made approximately 1,100 doses of 'PPSB' from blood collected in EDTA, by the method of SOULIER *et al.* [20]. This product represented an important advance in the treatment of haemophilia B, and its use has been extended to an increasing number of other deficiencies of the prothrombin complex of coagulation factors [5]. The demand for PPSB for use, e.g., in liver disease and the reversal of anticoagulant therapy, at times threatened to exhaust the stocks required for emergency treatment of haemophilia B, and prompted us to look for

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new methods of recovering factor IX or prothrombin complex from normal citrated plasma. A large increase in the production of PPSB was considered uneconomical of limited fresh blood resources, because cellular components and factor VIII are not readily recovered from blood collected in EDTA. It was hoped that an alternative method could be devised to improve the yield of factor IX (only about 30 % in large-scale production of PPSB) and end our dependence on procuring batches of tricalcium phosphate with the appropriate absorptive properties.

This report describes the absorption of factors II, IX and X from large batches of citrated Cohn supernatant I on DEAE-cellulose, and their selective elution in a form suitable for freeze-drying and administration to patients without further purification or stabilisation. Some of this work has been described in a preliminary form [19].

Several plasma fractionation laboratories are currently using anion exchangers to prepare factor IX concentrates. Efficient absorption of prothrombin complex from citrated plasma on a preparative scale was first achieved in the Central Laboratory of the Netherlands Red Cross [7]; their method of pre-cycling DEAE-cellulose has been adopted in this work without modification [15]. The principle of batch absorption of factor IX, followed by column elution, was first applied successfully, on a rather smaller scale, by CASILLAS *et al.* [6].

The wide range of alternative methods for the production of factor IX concentrates has been well reviewed recently [8, 16].

Methods

Standard Procedure for the Preparation of Factors II, IX and X from 120 l Plasma

– *Preparation of ion-exchanger.* Unless otherwise stated, we refer to the dry weight of the exchanger. 1 kg DEAE-cellulose, Whatman DE.32, after hydration has a settled volume of approximately 5 l and a moist weight of approximately 2 kg when drained under gentle suction.

500-gramme batches of DE.32 were prepared in an MSE 3000 basket centrifuge fitted with a perforated stainless steel rotor and a nylon filter bag. Fines were removed by at least two decantations from 10 vol of pyrogen-free distilled water before pre-cycling and equilibrating the DE.32 with the following reagents: 5 l 0.5 N HCl, followed by water to pH >4; 5 l 0.5 NaOH, followed by water to pH <8; 5 l 1.0 N NaCl, followed by water to conductivity <1 μmho.

3 l of each reagent was added to the exchanger with the rotor in motion. As a precaution against channelling, the remaining 2 l was added with the rotor at rest, so that the exchanger bed collapsed in the reagent. After 30 min, the reagent was washed out with a large volume of pyrogen-free distilled water (approximately 30 l) while the rotor operated

at approximately 200 *g*. After the final wash, the exchanger was transferred to 1 l polypropylene jars, autoclaved at 120°C for 20 min, and stored at 5°C.

Preparation of plasma. Cryo-precipitate supernatant from blood collected in ACD anticoagulant was frozen and stored in PVC or polyethylene bags or in glass bottles for several weeks. This plasma was thawed the day before the preparation of the concentrate, and Cohn fraction I removed after overnight equilibration at –2°C, pH 6.8 and 8% ethanol.

Absorption. 120 l supernatant I was mixed with 40 l pyrogen-free distilled water to reduce its ionic strength, and the temperature of the diluted liquor maintained at 2 ±1°C in a cold room. 1 kg prepared DE.32 was stirred vigorously with this liquor for 90 min, then removed by passage through a Sharples supercentrifuge at 120 l/h. Centrifugation was continued for at least 30 min after the last of the suspension had entered the centrifuge. This minimised the loss of DE.32 in the suspension which fell out of the half-filled rotor when it came to rest. The supernatant was further processed to recover IgG and albumin.

Buffer solution. The wash buffer solution, containing 0.03 M phosphate, 0.03 M citrate, was prepared from analytical grade sodium dihydrogen phosphate, trisodium citrate and sodium hydroxide, adjusted to pH 6.9 ±0.1, conductivity 9 mmho, ionic strength 0.24. Eluting buffer contained 0.03 M phosphate, 0.03 M citrate and 0.2 M NaCl, pH 6.9 ±0.1, conductivity 20 mmho, ionic strength 0.44. All buffers were made freshly from pyrogen-free distilled water only a few hours before use.

Column elution. For convenience, washing and elution were carried out at room temperature using buffers pre-cooled to 5°C. The exchanger, with its absorbed and entrapped proteins, was stirred to a smooth slurry with 4 l wash buffer, and poured into a cylindrical filter whose outlet was fitted with a flow-through conductivity cell (Radiometer CDM 2e). Two filters used with equal satisfaction were [1] an elongated sintered glass funnel, porosity 1, diameter 150 mm and [2] a column assembled from 225 mm glass pipeline (James J. Jobling & Co. Ltd., Trentham, Stoke-on-Trent, England) and a 200 mesh stainless steel filter. Both filters with their associated tubing, etc., were autoclaved before use. While still draining, the bed was protected by a floating sheet af glass or polypropylene fabric. When the bed had drained to form a squat column, interstitial protein was washed through with 4 l of the same buffer, before 6 l of the eluting buffer was added. The emergence of the coagulation factors, immediately after the 'salt front', was marked by a sharp rise in conductivity. Six or more successive 400-ml fractions were taken following this. After sub-sampling for factor IX and other assays, these fractions were frozen.

In a few runs, the flow was assisted by gentle suction from a peristaltic pump, but the total time for washing and elution, even under gravity, was usually less than 2 h. After elution, strongly bound proteins were eluted with 6 l 1.0 M NaCl, and the exchanger was frozen before being re-cycled in precisely the same way as new exchanger.

Preparation for clinical use. Fractions of eluate containing factor IX were selected to form a pool containing approximately 30 U/ml. These fractions were thawed, pre-filtered immediately through Cox or Millipore membrane filters, and finally filtered through a 0.2 or 0.22 μm autoclaved filter into a sterile receiver. From this receiver 10-ml doses were filled into 30-ml vials, which were rapidly fitted with gauze caps and frozen prior to freeze-drying. Drying was continued for 24–48 h without heat, so that the temperature of the product never exceeded 15°C. The final vapour pressure in the chamber was less than 0.005 Torr. After drying, vials were rapidly sealed under ambient conditions before storage at 5°C.

Analytical methods. Total protein was determined by the biuret method of GORNALL *et al.* [10]. Sodium was determined by flame photometry. Chloride was determined by a titrimetric method (Sigma Kit No. 830). Citrate was determined by the method of SAFFRON and DENSTEDT [17] and phosphate by the method of YOUNG [23]. Conductivity was measured using a Radiometer CMD 11 electrode; pH was measured, at room temperature unless specified, using a combined glass electrode and a Radiometer PHM 26 meter. Cellulose acetate electrophoresis was carried out on Gelman Seprophore III medium at pH 8.6 in barbitone buffer. Polyacrylamide gel electrophoresis was carried out in thin vertical slabs [1] at pH 8.6 in 7.5% acrylamide gel.

Coagulation factor assays. Factor IX was determined using a one-stage kaolin-cephalin assay [12]. Factor VII was measured by the method of GARNER and CONNING [9], using factor VII-deficient dog plasma. Factor II was measured using tiger-snake venom [14]. Factor X was measured using Diagen factor X substrate plasma (Diagnostic Reagents Limited, Thame, England).

Determination of thrombin and thromboplastin-like substances. Attempts were made to measure the thrombin content of the factor II, IX, X concentrate by incubating it, with or without calcium, with either a 1-percent solution of fibrinogen (Kabi, >95% clottable) or with fresh citrated plasma. Fresh dilutions of Parke-Davis bovine thrombin from 1.0 to 5×10^{-4} NIH U/ml were used to calibrate each test. Siliconised glassware and disposable polyethylene pipette tips were used throughout. Mixtures were incubated at 20 or 37 °C, and the first development of fibrin threads or gel recorded after direct observation.

Results

Elution Pattern

Factors II and X were eluted together with factor IX and protein (fig. 1). The small amount of factor VII absorbed from the specified dilution of supernatant I was eluted by the wash buffer, and the concentration of factor VII in the final product was always less than 0.5 U/ml.

Since factors II, IX and X were eluted on the leading edge of the salt front, the total salt content of the product was less than that of the eluant. The chromatographic system contained many anions, including proteins, chloride, phosphate and citrate. Higher concentrations of citrate and phosphate were present in the product than in the eluant, since these buffer ions replaced some protein during the first wash, and were then displaced by chloride during elution proper. Very little chloride, which displaced all other anions from the exchanger during elution, appeared in the product.

Description of the Dried Product

The white powder redissolved very readily in 10 ml water to a faintly opalescent solution, sometimes containing a few threads of denatured fibrinogen formed in freezing or drying.

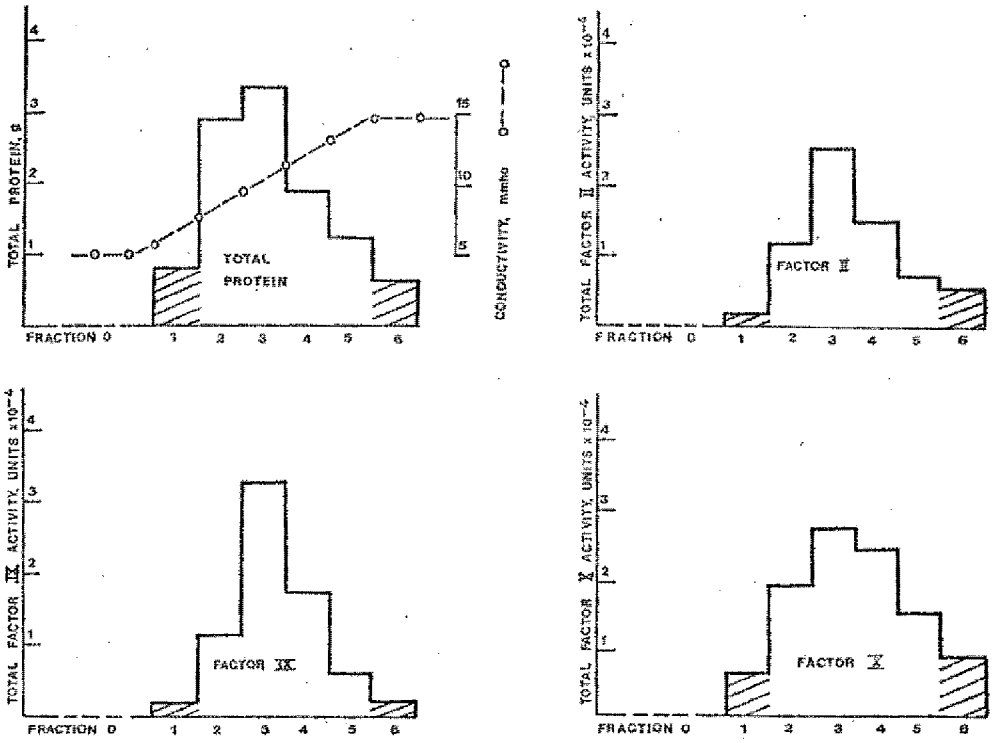

Fig. 1. Frontal elution of protein and factors II, IX and X from ion-exchanger. Following the addition of eluting buffer to the column, fraction O (approximately 1.5 l) was discarded. At the first indication of a rise in conductivity of the eluate, fractions 1–6 (each 400 ml) were collected. The conductivity of the eluate was recorded at intervals and the total of amounts of factors II, IX and X and protein in each fraction were measured.

The product so dissolved contained 210–280 mM Na^+, 40–90 mM citrate, 20–50 mM phosphate and 5–20 mM Cl^-. The conductivity was approximately 13 mmho and the osmolarity, as measured by freezing point depression, approximately 320 mOsm. The pH was 6.8–7.1 and the protein content 0.6–1.2 g%. Anti-A and anti-B were present at a titre of approximately 1/8. In terms of units of factor IX per mg protein, the product was approximately 250–300 times purified from supernatant I. Factors II and X were present in concentrations approximately equal to that of factor IX. Two-stage factor IX assays were consistently higher than one-stage assays, by approximately 50 %.

On electrophoresis in polyacrylamide gel, the concentrate was found to contain all the zones present in the preparation of DIKE *et al.* [8] with some differences in the relative intensities of the minor bands; our concentrate contained less pre-albumin, albumin and α_1-globulin than did that of DIKE *et al.* [8].

Recovery of Factor IX

The recovery of factor IX was calculated from the first 20 consecutive runs using the standard procedure. The mean recovery of the total eluted factor IX from supernatant I (mean factor IX content 0.71 U/ml) was 79 % (range 42–105 %). Of the total eluate, only fractions containing more than 10 U/ml were usually selected for pooling and sterile filtration, in order that the pool should contain more than 30 U/ml factor IX; the mean yield of this 'selected' eluate was 72 %. Since the preparation was made from readily available citrated plasma, the yield was not of primary importance.

Stability

Occasionally, losses up to 25 % of factor IX activity were noted following repeated freezing and thawing of the eluate. This may have been due to unrecorded differences in freezing rate, storage temperature, or method of thawing. Similarly, a long period of frozen storage of the preparation prior to lyophilisation occasionally resulted in incomplete recovery of factor IX activity and, in one case, in a slight increase in thrombin content. While freezing, filtration and lyophilisation have normally resulted in virtually complete recovery of factor II, IX and X and no increase in thrombin content, it is advisable to freeze the solution as rapidly as possible, thaw in a water bath with gentle stirring at 20–30 °C, and keep the preparation in the frozen state for a minimum time before lyophilisation.

After reconstitution from the dry state, the thrombin and factor II, IX and X content of the preparation did not change significantly over at least 6 h at room temperature. Eight randomly selected bottles of the lyophilised concentrate, stored from 11 to 26 months at 5 °C, showed a mean loss of 0.25 % of initial factor IX activity per month. This loss is less than the probable variation of coagulation factor assays over long periods.

Thrombin-Like Activity

When 0.1 ml thrombin was added to 0.1 ml of 1 % fibrinogen in the presence of 0.1 ml 50 mM calcium chloride, the end-point was difficult to discern at low concentrations of thrombin. The incubation of 0.1 ml dilute

thrombin standard and 0.1 ml 1% fibrinogen without calcium, was preferred since the longer clotting times offered greater discrimination, and since the formation of fibrin threads was more distinct; this latter procedure will be referred to as the thrombin-fibrinogen test. Using this test, the thrombin content of seven randomly selected batches of concentrate lay between 10^{-3} and 10^{-2} NIH U/ml.

When citrated plasma was used as a source of fibrinogen, without the addition of calcium, the clotting times for all batches of factor II, IX, X concentrate, and all concentrations of thrombin below 10^{-2} NIH U/ml, were greater than 24 h. It was found more useful to add 0.1 ml 50 mM calcium chloride to 0.1 ml plasma and 0.1 ml factor II, IX, X concentrate (or thrombin) in a 'recalcification' test, where the control time in the absence of thrombin was approximately 120 sec. Using this plasma recalcification test at 37 °C, the addition of the concentrate lengthened the clotting time, usually by 10–100 sec; the first fraction of the factor IX-containing eluate usually decreased the recalcification time by up to 10 sec, but subsequent eluate fractions increased the recalcification time. It was confirmed that lengthening of the recalcification time was primarily due to the citrate and phosphate ions eluted with the coagulation factors.

Pyrogenicity in Rabbits

Every batch of concentrate has been tested for pyrogenicity by the infusion of 3 ml/kg into the ear vein of three rabbits, at a rate not exceeding 10 ml/min. The maximum temperature rise during a period of about 3 h was measured, rather than a temperature rise at a fixed time after infusion. The Protein Fractionation Centre has had considerable experience in interpreting pyrogen tests on distilled water, human IgG, albumin and other solutions for injection. For such solutions a mean temperature rise of 0.2 °C is not considered significant; a mean temperature rise between 0.2 and 0.5 °C indicates the probable presence of bacterial pyrogen in amounts unlikely to cause observable fever in patients; solutions giving a mean rise greater than 0.5 °C usually result in a marked pyrogenic reaction in most patients. There is no clear linear relationship between the amount of bacterial pyrogen administered and the subsequent temperature rise.

Almost every batch of factor II, IX and X concentrate has caused a mean temperature rise of 0.5–1.5 °C in rabbits. The progress of the temperature rise has not differed significantly from that observed following the infusion of standard bacterial pyrogen, or pyrogenic albumin. In a single experiment, the temperature rise due to the infusion of a mixture of standard bacterial

pyrogen and concentrate was approximately equal to the sum of the rises due to the infusion of each component separately. Despite this exceptionally high pyrogenicity in rabbits, with the single exception discussed below, the concentrate has not caused pyrogenic reactions in patients.

Batches of concentrate were made according to the method of DIKE *et al.* [8], or using the latter's buffer system in preparations otherwise identical with our standard procedure. All proved as pyrogenic in rabbits as our routine batches, but those issued for clinical use were not pyrogenic in human patients. When separate portions of the eluate peak were tested, it was found that pyrogenicity correlated well with protein and factor IX concentrate of each sub-fraction, indicating that the problem could not be avoided by sharper chromatographic resolution.

Compared with most plasma fractionation techniques, the preparation of factor II, IX and X concentrate is rapid and uses simple apparatus and safe reagents, but it was considered important to eliminate the possibility that bacterial pyrogen was being formed during the preparation, or transferred from contaminated reagents or apparatus. Several batches of the citrate-phosphate-saline eluant were found to contain negligible pyrogen. The aqueous supernatant from autoclaved DEAE-cellulose immediately prior to absorption was apyrogenic, but pyrogen might have remained bound to it. The pyrogenicity of the product was not associated with the use of new exchanger or the repeated re-use of the same batch of exchanger.

Several batches of DE.32, some completely fresh and some which had been used for several preparations of factor II, IX and X concentrate, were used in control experiments which completely duplicated the standard preparation of the concentrate, except that pyrogen-free distilled water replaced plasma in the first stage. The eluate fraction, detected by conductivity changes, was sterile filtered, freeze-dried and pyrogen tested exactly as if it had contained coagulation factors. All such control preparations were apyrogenic, firmly implicating plasma as the source of pyrogenicity in rabbits.

In an extension of these control experiments, the supernatant from a routine absorption with DE.32 was immediately reabsorbed on a second batch of DE.32 and a 'second eluate' obtained in the normal way; selection of the second eluate was assisted by the presence of residual factor IX eluted in the normal position. The eluates from the first and second absorptions were sterile filtered, dried and pyrogen tested under identical conditions. The mean temperature rise (3 rabbits) for the first eluate was 1.6 °C, and for the second eluate (5 rabbits) 0.5 °C.

A search was made for substances other than bacterial pyrogen which

might cause pyrogenic reactions in rabbits. It has been shown by GREISMAN and HORNICK [11] that human plasma contains concentrations of heteroagglutinin which may be lethal or cause severe pyrogenic reactions in rabbits and other animals. Heteroagglutinin tests on the factor IX preparation and a variety of other plasma products were carried out using fresh and glutaraldehyde-treated rabbit erythrocytes [13]. Comparable titres were found for the factor, II, IX and X concentrate and pooled normal plasma. This result alone does not explain the pyrogenicity of the concentrate, since other apyrogenic plasma fractions such as IgG and PPSB had similar or higher heteroagglutinin titres. The relevance of immunoglobulin type to heteroagglutinin activity and pyrogenicity is being pursued in the light of these results.

It has been found that IgM, and rather less IgG, is eluted coincidently with the factor IX-containing fractions, using either our standard procedure or the conditions of DIKE *et al.* [8]. The 'second eluate' from the control experiment discussed above contained less IgM than the 'first eluate'. It is possible to speculate that the pyrogenicity in rabbits reflects a species-specific property of an immunoglobulin or other contaminant of the coagulation factors.

Animal Safety Tests

Routine safety tests have been carried out in young guinea pigs as an adjunct to sterility tests. After injection of 2 ml intraperitoneally, normal health and growth were maintained for more than 1 week. The pyrogen tests discussed above also constituted an acute toxicity test [3] at a dose exceeding the maximum required human dose of 2 ml/kg, or 60 units of factor IX/kg. No rabbit has died as a result of the injections. The only feature distinguishing the factor II, IX and X concentrate from other solutions has been a higher incidence of restlessness during injection. Respiratory distress and clotting in the injected veins have been reported occasionally in individual rabbits but such reactions have not been consistently related to any batch of concentrate and are not unknown in the pyrogen testing of other solutions. Rabbits killed within 30 min of injecting up to 10 ml/kg have shown no gross or microscopic evidence of changes in kidney, lung, heart or vascular tissue.

As part of a wider investigation to be reported separately, doses up to 5 ml/kg have been infused into dogs in attempts to detect intravascular coagulation. It was concluded that, at this dosage in the dog, the factor II, IX and X concentrate provoked some intravascular coagulation, but less than did the Protein Fractionation Centre's preparation of PPSB.

Clinical Safety

The first trial infusion of 100 ml of this concentrate elicited a typical reaction to bacterial pyrogen; the patient's temperature rose by approximately 2 °C over 3 h, the reaction being alleviated by the administration of hydrocortisone. This batch of concentrate was later found to have been in contact with contaminated surfaces in the Sharples centrifuge. Since this fault was remedied, there has been no recurrence of febrile reactions in this or any other patient.

More than 1,200 doses have been administered to more than 30 patients; some patients have received more than 50 doses in less than a week, and some have received several doses per week for more than 18 months. Clinicians have been warned specifically of the possibility of pyrogenic reactions, but none has been reported. Patients have experienced no discomfort near the site of injection, and the slight hyperosmolarity of the preparation has caused no difficulty. No other adverse reaction has been reported. A cirrhotic patient who received 60 ml of the preparation prior to minor surgery subsequently showed no clinical or laboratory signs of intravascular coagulation. Since detoxification of activated factors might be expected to be impaired where liver function is deficient, this uneventful infusion suggests that the preparation may not contain dangerous amounts of activated coagulation factors.

Haemostatic Response

No case has been reported of failure to achieve haemostasis by administration of the calculated dose. Clinicians have usually adopted a dosage of 30–50 U/kg during active haemorrhage, followed by maintenance with 20–30 U/kg. A more objective measure of the response is derived from the rise of plasma factor IX concentration following treatment. The dose response can be defined [3] as the rise in plasma factor IX per unit of factor IX in the dose, multiplied by the patient's weight in kg, i.e.

$$\text{Dose response} = \frac{\text{rise (\%)} \times \text{body weight (kg)}}{\text{units factor IX administered (U)}}$$

Useful data have been obtained from a total of 42 infusions (totalling 218 vials) of doses ranging from 20 to 60 U/kg. The rise in plasma factor IX in each case has been reported by the clinician's own laboratory, while the dose administered has been calculated from our own data on the issued product. The results of these infusions will be only broadly summarised,

since others may wish to discuss individual cases in the more detailed context of patient care.

The range of dose responses, as defined above, over all infusions has been 0.22–1.5 (mean 0.69). There has been no significant difference in responses to different batches, or to first and subsequent doses, although some first doses have been administered during active bleeding. The range of response of each patient to separate infusions has been almost as wide as the range of all responses of all patients. The half-life of administered factor IX in the patient has been in the range of 24–48 h.

Transmission of Serum Hepatitis

More than 20 batches, each made from 200 to 600 donations of plasma, have been used since May 1970. In Scotland, screening of all donations of blood for Australia antigen by immunodiffusion or immunoelectroosmophoresis became routine during 1971. Using such methods, which detect probably less than 50 % of Australia antigen carriers, the incidence of antigenaemia among blood donors in Scotland has been found to be about 0.07 % [21]. All batches of the factor II, IX and X concentrate have been tested for Australia antigen by the methods used for donor plasma, usually in five-fold concentration. Australia antigen has not been found in any batch, nor has any recipient developed hepatitis or Australia antigenaemia following treatment with the concentrate alone.

Since the methods used to detect the agent causing serum hepatitis were very insensitive and since no systematic attempt has been made to assess the incidence of sub-clinical hepatitis in the recipients, it would be prudent to assume that the concentrate could be infective if made from infective plasma, but it seems likely that the concentration of the infective agent is substantially reduced by the preparative procedure.

Variations from Standard Procedure

After more than 20 batches of the standard preparation had been made, some of the conditions were varied in attempts to simplify the procedure, improve recovery of factor IX even further, or modify the product's pyrogenicity in rabbits.

Preparations described in the present paper used 1 kg DE.32 in 160 l diluted plasma, which offered a comfortable excess of DE.32 and allowed repeated use of a single batch of exchanger despite 5–10 % loss during each cycle; a 1-kg batch could be used until its settled volume was reduced by about 30 %, or the lost material could be replaced with 100 g of new ex-

changer after each cycle. When the proportion of DE.32 was reduced to 500 g in 160 l diluted plasma, the absorption of factor IX was almost as good, provided that very efficient mixing was achieved during absorption. The volumes of wash and eluting buffer could be reduced, and factor IX eluted at a higher concentration than in the standard procedure, but the product was otherwise unchanged. If the results of clinical trial are equally good, this reduced proportion of DE.32 will be adopted routinely.

The ion-exchanger DE.52 is claimed by the manufacturers to be similar to DE.32, except that it has never been dried. DE.32 was originally selected for this preparation on the ground that, as a dry powder, it was less likely to encourage bacterial growth. Two runs were carried out using DE.52 in place of DE.32. This batch of DE.52 proved more difficult to centrifuge from the plasma, but the product was indistinguishable from that made using DE.32.

If DE.32 was pre-cycled as usual with acid and alkali, but omitting the wash with 0.5 M NaCl, the exchanger could be used in the free base (hydroxyl) rather than the chloride form. In our hands, the use of DE.32 (either 500 g or 1 kg in 160 l diluted plasma) as the free base resulted in slightly lower average yields of factor IX; although the product was otherwise very similar to that obtained with DE.32 in the chloride form, the latter form was preferred.

In attempts to reduce the loss of DE.32 through the creation of fine particles during vigorous stirring with the plasma, a variety of propellors and dispersers were used, running continuously or intermittently. It was concluded that, particularly if less than the standard proportion of ion-exchanger were used, some loss of DE.32 was preferable to inefficient mixing. The plasma and exchanger are now mixed continuously for 90 min in a 200-litre steel vessel, using a 4-inch stainless steel marine propellor rotating at 1,500 rpm. Since 5–10 % DE.32 falls out of the Sharples continuous-flow rotor after each centrifugation, attempts were made to reduce this loss of exchanger and absorbed coagulation factors by substituting other methods of collecting the exchanger. In theory, a basket centrifuge could be used to collect the loaded exchanger which could then be washed and eluted while still in the rotor. However, the exchanger loaded with protein offers considerable resistance to flow, and the capacity of the MSE 3000 basket centrifuge was too small for this purpose. While washing the loaded exchanger in the rotor would be attractive, the coagulation factors may be eluted frontally in a much higher concentration than is possible using batch elution.

Several attempts were made to modify the buffers used to wash and elute the loaded exchanger. The function of the wash buffer is to remove as much as possible of the inert protein, while retaining coagulation factors II, IX and X on the exchanger. The wash solution must contain enough buffer ions to control pH, and must have maximum calcium-binding capacity to minimise activation of the coagulation factors. Citrate has strong calcium-binding properties at neutral pH, but its poor buffering capacity permits the pH of the eluate to drift above 8. Phosphate buffers well, but its low calcium-binding capacity may allow small clots to form in the column or eluate. A buffer containing 0.03 M citrate and 0.03 M phosphate at pH 6.8 has sufficient buffering and calcium-binding capacity, and a low enough ionic strength to permit retention of factors II, IX and X on the exchanger. Wash buffer containing 0.02 M citrate, 0.02 M phosphate (ionic strength 0.16) is just sufficient to control pH and calcium. If the ionic strength of the latter wash buffer is increased to that of the 0.03 M citrate, 0.03 M phosphate buffer by adding 0.08 M NaCl, washing efficiency is maintained without any significant difference in the subsequent pattern of elution.

The substitution of chloride for an even larger proportion of the citrate and phosphate was investigated in several large-scale experiments. One experiment was carried out precisely according to the procedure of DIKE *et al.* [8] using a 10 × 60 cm column; in other experiments all our standard conditions were used except for the substitution of the exchanger equilibrating conditions and elution buffers of DIKE *et al.* [8]. Differences in column dimensions had little effect on the distribution of proteins in the eluate, although the sharper elution made possible by a narrow column yielded a higher ratio of factor IX to salts. The lower content of citrate and phosphate led to difficulty in maintaining close control of pH, and may have contributed to the losses of activity noted by DIKE *et al.* [8] during freezing and lyophilisation of the eluate.

None of these modifications significantly altered the eluate's pyrogenicity in rabbits.

Discussion

The preparation has been designed to be very simple and rapid and to cause minimal interference with a fractionation centre's commitment to the large-scale production of other plasma protein fractions. The preparation works well on a 1-litre scale, and should be applicable up to at least 1,000 l without difficulty, since the design and dimensions of the eluting column are

not critical and the volume of absorbent is quite small. The method does not require automated elution equipment.

The thrombin content of the preparation, as measured in the thrombin-fibrinogen reaction, is greater than the limit (10^{-3} NIH U/ml) tentatively set by the US National Institutes of Health. We are not aware of any publication ascribing undesirable physiological effects to such small amounts of thrombin. The presence of citrate and phosphate ions in the preparation minimises the hazard of residual thrombin activity and, if it proves necessary, the addition of heparin to the preparation could provide an additional safeguard.

WICKERHAUSER and SGOURIS [22] have shown that it is possible to suppress the activity of thrombin and factor Xa in a preparation of prothrombin complex by the addition of heparin and heparin cofactor prepared from Cohn fraction IV. It is not certain that this offers complete protection to the patient receiving the preparation, and the risk of transmitting serum hepatitis is increased by the inclusion of fraction IV derivatives [2, 18].

After the infusion of more than 1,000 vials of the factor II, IX and X concentrate over 2 years, it has been found safe to use in high and repeated doses, and at least as effective as any other concentrate in maintaining haemostasis in haemophilia B. The simplicity of the preparation and its use of readily available grades of citrated plasma should ensure for the first time in Scotland an adequate supply of factor IX concentrate for the emergency treatment, and even prophylaxis, of all patients with haemophilia B.

It is not yet known to what extent this factor II, IX and X concentrate can be substituted for PPSB or other sources of factors II, VII, IX and X, in, e.g. cirrhosis or anticoagulant reversal. When it first became clear that the preparation would be greatly simplified and its stability improved by the omission of factor VII, it was considered that factor VII deficiency would only very rarely limit haemostasis. Until those classes of patient requiring factor VII replacement have been accurately identified, limited supplies of expensive factor II, VII, IX and X concentrate should be used much more discriminately.

The new concentrate is not free of disadvantage; most important is the difficulty of interpreting the anomalous results of rabbit pyrogen tests. If the concentrate is to be used clinically, despite this difficulty, even more stringent precautions than usual must be observed to avoid formation of bacterial pyrogen, which might be masked by the apparently species-specific pyrogen. The effective loss of one of the most important tests for clinical safety is a major restraint on the broader application of the concentrate, and an explanation of the phenomenon is being sought urgently.

Dilution of the plasma with one third of its volume of water increases the cost of subsequent ethanol fractionation of the supernatant. We could not achieve complete absorption of factor IX from undiluted plasma [6]; absorption was improved at higher temperatures, but the penalty of dilution was preferred to the risk of activating the coagulation factors.

There is no reason to believe that the concentrate cannot transmit serum hepatitis; however it is probably better in this respect than alternative sources of factor IX, including plasma. The factor IX content of the preparation, diluted to iso-osmolar solution, is too low for convenient administration by syringe in the most severe haemophilic emergencies. We hope to achieve an acceptable solution to the latter two difficulties by further treatment of the eluate.

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