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(54) Title: PURE FACTOR IX PRODUCT

(57) Abstract

A pure Factor IX (FIX) product isolated from plasma or plasma concentrates is described. This protein is free of other human proteins. No other detectable coagulation factors are present. In addition, an immunoaffinity process is presented which shows no leakage of murine monoclonal antibody.

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PURE FACTOR IX PRODUCT

BACKGROUND OF THE INVENTION

Technical Field:

The present invention is related to the preparation of pure Factor IX product from human plasma or plasma concentrates. More particularly, the present invention is related to the pure preparation of Factor IX product free from all detectable thrombogenic factors and any other contaminating human plasma protein.

State of the Art:

Pure, non-thrombogenic preparation of Factor IX is used as replacement therapy in patients with haemophilia B. Although the product described in U.S. Patent 4,447,416 represented a 250-fold purification of plasma-derived Factor IX, the final product contains only ten percent Factor IX, has greater than trace amounts of the clotting factors IX and X and may also contain trace amounts of Factor VII.

Concentrates rich in Factor IX (FIX) were first prepared by adsorption of plasma by tricalcium phosphate followed by the elution of Factor IX, along with Factor II (prothrombin), Factor VII and Factor X (Didisheim et al, J. Lab Clin. Med. 53:322-330, 1959; Blatrix et al, Pathol. Biol. 7:2477-2486, 1959). Although a variety of fractionation procedures are known, all purification

methods take advantage of the common specific adsorbability of the vitamin K-dependent clotting factors for the preparation of these concentrates which are referred to in the literature by the generic names of Factor IX Complex, Prothrombin Complex or Factor IX concentrates.

In addition to Factor IX, all commercially available Factor IX concentrates contain substantial amounts of Factor II (Prothrombin) and Factor X and a variable amount of Factor VII. The Factor VII content depends upon the fractionation procedure, for example concentrates derived from calcium phosphate adsorption contain substantial amounts and those prepared by ion-exchange chromatography contain less (White et al, Blood 49:159-170, 1977). The potency of Factors II, IX and X is unaffected by Factor VII content (Menache et al, Patent No. 4,447,416, 1984).

It has been reported that although clinically efficacious, the use of Factor IX Complex concentrates, particularly when given in large amounts over a protracted period, produces adverse reactions of thrombosis and/or disseminated intravascular coagulation (DIC) (Kingdon et al, Thrombos. Diathes. Haemorrh. 33:617-631, 1975). Reports of complications following administration of Factor IX Complex concentrate to hemophilia B patients include thrombophlebitis at the site of infusion (Loeliger et al, Folia Med. Meerl. 10:112-125, 1967), superficial vein thrombosis (Marchesi et al, N. Engl. J. Med. 290:403-404, 1974), deep vein thrombosis, pulmonary embolus (Kasper N. Engl. J. Med. 289:160, 1973) acute myocardial infarction (Steinberg et al, N. Engl. J. Med. 289:592, 1973), and DIC (Edson N. Engl. J. Med. 290:403, 1974 and Schimpf et al, Thromb. Res. 8:65-70, 1976).

The nature of the thrombogenic material(s) in Factor IX Complex concentrate is unkown. Some studies have implicated the activated factors Xa, IXa, VIIa, or the contact phase factors (Hultin, Blood 62:677-684, 1983 and Seligsohn et al, Blood 53:828-837, 1979). Others have shown that the thrombotic complications are mediated by a combination of coagulant-active phospholipid and activated clotting factors (Giles et al, Blood 59:401-407, 1982). Experiments in mice suggested the thrombogenicity was due to an induced zymogen overload, particularly factor II and factor X which have relatively long half-lives and could accumulate in the circulation (Magner et al, Devel. Biol. Stand. 44:185-188, 1979). A recent report suggests that ATIII changes which occur predictably after commercial Factor IX Complex concentrate therapy are caused by a contaminating protein or proteins other than factor IX and that these ATIII changes may be related to the thrombotic complications of the commercial concentrates (Hoffman et al, Thromb. Res. 43:143-151, 1986).

Though the exact nature of the thrombogenic material in FIX preparations is not known, preparations of highly purified material will potentially eliminate all other contaminants and be non-thrombogenic by this criterion. In an effort to make a purer product, immunoaffinity techniques have been developed. In general, immunoaffinity procedures for protein purification depend upon the generation, purification and subsequent production of a monoclonal antibody (MAb) to the protein of interest. The MAb must then be covalently bonded to a solid matrix without disruption of the MAb-protein binding site. This then constitutes the ligand-substrate matrix which can be utilized for the purification of the protein of interest.

Several investigators have prepared MAbs to clotting factors in general (Jenny et al, Prep. Biochem. 16:227-245, 19861; Corssant et al, Thrombosis and Haemostasis 56:271-276, 1986) and the FIX in particular (Liebman et al, PNAS 82:3879-3883, 1985; Bessos et al, Throm. Res. 40:863-867, 1985; Smith et al, Throm. Res. 33:211-224, 1984; and Thompson Blood 62:1027-1034, 1983). In addition, polyclonal antibodies have also been used (Bessos et al, Thrombosis and Haemostasis 56:86-89, 1986). However, all procedures involving FIX MAbs reported so far, require extremely harsh elution conditions to dissociate the FIX, resulting in low yield of FIX and low reusability of the monoclonal antibody column. In addition, the product obtained, albeit quite pure in comparison to plasma protein concentrates, still remains contaminated with about 1% of Factor II and X and about 3% of murine IgG (Bessos, et al, supra. 1986).

SUMMARY OF THE INVENTION

It is, therefore, an object of the present invention to provide a pure coagulation factor IX (FIX) product devoid of detectable levels of other common contaminant proteins found in heretofore obtained preparations of FIX.

Other objects and advantages of the present invention will become evident from the following detailed description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

These and other objects, features and many of the attendant advantages of the invention will be better understood upon a reading of the following detailed

description when considered in connection with the accompanying drawings wherein:

Figure 1 shows chromatographic results obtained from the purification procedure in accordance with the present invention. The full circles represent protein concentration as measured by absorbance at 280 nm. The full triangles represent FIX activity as measured by a single stage clotting assay as described in Example 2 herein;

Figure 2 shows the characterization of monoclonal antibody-purified FIX. Western blots against Factors II, IX and I against Protein C for the starting material and the MAb purified product. SDS polyacrylamide gel is also shown. Lanes are as described;

Figure 3 shows the characterization of monoclonal antibody purified FIX. Western blots against inter-alpha-trypsin inhibitor, factor IX and murine IgG. Commassie Blue stained SDS polyacrylamide gel is also shown, including standards. The antibody to murine IgG was shown previously to be reactive against mouse IgG; and

Figure 4 shows the results of HPLC of monoclonal antibody purified FIX product. B was increased to 80% as explained in Example 4. The full scale corresponds to an absorbance at 211nm of 1.0.

DETAILED DESCRIPTION OF THE INVENTION

The above and various other objects and advantages of the present invention are achieved by an isolated, pure Factor IX product free of any detectable plasma or coagulation factor impurities.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as

commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned hereunder are incorporated herein by reference.

MATERIALS AND METHODS

Monoclonal Antibody Production:

Male BALB/c mice were immunized with 3 consecutive injections of conventionally purified Factor IX (Bajaj et al, Preparative Biochemistry 11:397-412, 1981). The immunization schedule involved an initial intraperitoneal injection of the purified antigen (50 ug) in complete Freunds adjuvant, followed by a intraperitoneal injection of purified antigen (50 ug) in incomplete Freunds adjuvant after 2 weeks. Following the second injection, the mouse was bled and the titer determined. A third intraperitoneal injection, consisting of purified antigen (50ug) in saline, was given at week 4. Three days after the final injection, spleen cells (1×10^8) from immunized mice were fused with the mouse myeloma cell line (1×10^7) P3-X63-Ag8.653 (Kearney et al, J. Immunology 123:1548-1550, 1979) in 50% polyethylene glycol 400 (Merck) by the standard procedure (Kohler and Milstein Nature 256:495-497, 1975). The fused cells were suspended in hypoxanthine- and thymidine- containing medium overnight (about 16 hours), then resuspended in hypoxanthine-aminopterin-, and thymidine-containing medium, and distributed into 8 microtiter plates. Supernatants were assayed after 15-20 days. The selected positive cultures were cloned by the standard limiting

dilution method with each cloning performed on wells producing antibody which were at average dilutions of one cell or less per well and which visibly demonstrated growth as a single clone of cells. All clones demonstrated the same properties as the parent cell line in the screening assay.

Antibody Screening Assay:

Cell supernants were assayed by using a solid phase enzyme-linked immunoabsorbent assay (ELISA). Purified human FIX was coated to the wells of microtiter plates (Immunolon 2, Dynatech) at a concentration of 1 mg/mL in 50 mM $NaHCO_3$, pH 9.5, overnight at 4°C. Following washing, the plates were coated with bovine serum albumin (10 mg/mL) in the same buffer for 1 hour at 37°C. The plates were washed and then incubated with cell supernatants in a 50 mM sodium phosphate buffer, pH 7.4 containing 0.15 M NaCl/0.02% Tween 20/10 mM $CaCl_2$. Binding of antibodies to the antigen-coated well was detected by adding an antimouse IgG-alkaline phosphate conjugate.

Characteristics of the Antibody:

The purified antibody contained a single band of approximately 150 kDa upon SDS-PAGE under nonreducing conditions. Analysis of the purified antibody by SDS-PAGE under reducing condition revealed that the molecule is comprised of two subunits, with approximate molecular weights of 50 kDa and 25 kDa, which is characteristic of the heavy and light chains of IgG. The purified antibody was subtyped by the Ouchterlony technique and identified as belonging to an IgG1 subclass with a kappa light chain.

Purification Process

The coupled gel is poured into a column. The column is equilibrated in buffer containing 10 mM Mg^{2+}. The sample to be applied can be any source of FIX including plasma, plasma protein concentrate and the like. The sample is equilibrated so that an excess of Mg^{2+} or Ca^{2+} is present and is then applied to the column. The column is then washed until the absorbance of the column effluent is less than 0.1. Any other means of detecting protein may also be utilized. Absorbance at 280 nm is chosen as an example of a relatively straight forward and direct method of on-line protein estimation. After the absorbance reaches a value <0.1, the washing buffer is changed to a buffer to chelate the metal ion. This may be an EDTA based buffer, a citrate based buffer or any other chelating buffer well known to one of ordinary skill in the art. The eluation results in a single protein peak as monitored by tracking the absorbance at 280 nm. The eluted protein is then assayed for FIX by activity assay, tested for purity using SDS-PAGE, N-terminal sequencing and western blotting. Other assay methods could also be used.

E X A M P L E 1

Purification of Factor IX from DEAE-Sephadex Eluate

For this purification, Sepharose CNBr4B was used as the immobilization substrate. 2.5 grams of freeze dried resin was suspended in 1mM HCl and washed for 15 minutes in 1mM HCl. 31 mls of protease inactivated (56°C for 30 minutes) monoclonal antibody solution (0.86 mg/ml protein, >90% IgG SDS-PAGE) was then mixed with the gel and allowed to mix end-over-end for 2 hours at room temperature. The gel was then washed with 20 mM PO_4,

100 mM NaCl, pH 8 and any remaining active groups were blocked with 200 mM glycine, pH 8 for 2 hours. The gel was then washed in turn with acetate buffer (0.1M, 0.5M NaCl pH 4) and phosphate buffer (20 mM, 100 mM NaCl, pH 8) in sequence several times. This resulted in coupling of 2.9 mg protein/ml of gel. The product was then stored in the cold in phosphate buffer containing 0.2% azide for further use.

The coupled gel was poured into an Amicon G10 x 150 Column, forming a bed of volume 7.5 cm^3. The column was equilibrated with equilibration buffer (10 mM $MgCl_2$, 100 mM NaCl, 20 mM TRIS, pH 7.5 with 0.2% NaN_3) using a flow rate of about 0.4 ml/min. The sample to be applied was an eluate from a DEAE Sephadex adsorption of cryo-poor plasma (Run # 283E016 from American Red Cross at Hyland Therapeutics). This sample was equilibrated to 40 mM $MgCl_2$ in order to balance to 20 mM concentration of citrate ion that the sample was in. The sample was then applied at the same flow rate of 0.4 ml/min giving a mean residence time in the column of about 18 minutes. Fractions were collected and assayed for protein content and factor IX clotting activity. The column was subsequently washed with washing buffer (10 mM $MgCl_2$, 1 M NaCl, 20 mM TRIS, pH 7.5 with 0.2% NaN_3) until the absorbance of the wash was below 0.02 at 280 nm.

At this point the buffer was changed to elution buffer (20 mM Sodium Citrate, 110 mM NaCl, pH 6.8 with 0.2% NaN_3). Elution fractions were also collected and assayed for protein and factor IX clotting activity. The profile of the adsorption, washing and elution are shown in Figure 1. Both the protein content and the clotting activity are included. Elution yielded a single protein peak as shown in Figure 1. The analysis in terms

of FIX specific activity and percent recovery is shown in Table 1.

TABLE I

Example 1: Purification of FIX from DEAE Eluate

Source Material	Volume (mls)	Total Factor IX Adsorbed/Eluted	RECOVERY %	U Factor IX/mg MAB	Specific Activity U FIX/ mg Protein
DEAE (E016)	9	909 U/906 U	99.8	38	248

The starting material was lot number 283E016 from the American Red Cross utilizing the pilot facility of Baxter Travenol at Glendale, California, which was eluate from a preliminary adsorption of cryosupernatant plasma on DEAE-Sephadex. The activity of Factor IX adsorbed and eluted was determined by one stage coagulation assays as described in Example 2. 1050 U FIX was loaded onto the column, and 141 U was present in the drop through. Total protein was determined by absorbance at 280 nm.

EXAMPLE 2

Factor Activity Tests

Factor IX activity was measured by a standard one-stage coagulation assay as described by Biggs (Human Blood Coagulation Haemostasis and Thrombosis ed. 1, Oxford: Balckwell Scientific, p614, 1972). Samples to be assayed are diluted 1:10, 1:20, 1:40, and 1:80 in a dilution buffer. The dilution buffer contains 0.05M Imidazole, 0.1 NaCl, pH 7.4 containing 0.1% w/v Bovine serum albumin (Sigma, RIA grade) and 0.01% v/v Tween 20 (Sigma). All samples are prediluted so that the FIX activity is below about 1 U/ml, corresponding to that of pooled plasma. The standard used was fresh frozen pooled plasma, consisting of a pool of not less than 10 donor units.

In the assay, 100 µl of FIX-deficient plasma (George King, Biomedical) and 100 µl of diluted sample were placed in a Coagamate X-2 tray (General Diagnostics) and the tray placed in a Coagamate X-2 coagulation machine. APTT Reagent (General Diagnostics) and $CaCl_2$ are then added to the test sample and the clot time is measured. The FIX activity in the same is then estimated by comparing clot times for standard plasma and the test sample. Factor II and X assays were performed similar to the assay for Factor IX except for two changes. The deficient plasma utilized was factor II and VII deficient (Sigma) for the Factor II assay and Factor II and VII deficient (Sigma) for the Factor X assay. Also, Russels Viper Venom (RVV, Sigma) and $CaCl_2$ were used instead of APTT and $CaCl_2$. These assays are quite standard in the art.

Protein C was assayed using a chromogenic substrate and the procedure outlined by Odegaard et al,

(Haemostasis, 17:109-113, 1987) as well as a coagulation assay (Francis et al, Am. J. Clin. Path. 87:619-612, 1987).

The results of the assay for Factor II, VII, IX, X and Protein C are shown in Table II. The data shows duplicate clot times measured as described and indicate quite clearly that clotting factors II, VII, X and Protein C are not detectable by any of the coagulation assays. Factor IX was determined independently since the concentration was too high and a 100-fold dilution was necessary to obtain meaningful results. A buffer clotting time is not provided because it is not useful for comparisons. In the Table, the clot time for Factors II, VII and X for the buffer are all less than the clot time for the monoclonal antibody purified product indicating the absence of any of these vitamin-K dependent coagulation factors in the product. The buffer clot time for Protein C is greater than the clot time for the monoclonal purified product, indicating the absence of any protein C in the product.

TABLE II

Example 2: Coagulation Assay Data

Component	Buffer Clot Time (sec)	MAb FIX Product Clot Time (sec)	Activity U/ml	Specific Activity U/mg
Factor IX	--	--	86.1	248
Factor II	180.5	190.8	0.0	-
		194.4		
Factor VII	9.3	9.5	0.0	-
		9.3		
Factor X	52.7	54.7	0.0	-
		54.2		
Protein C	39.0	34.5	0.0	-

Comparison of clot times for buffer and monoclonal antibody purified FIX product. Clot times were determined as described in Example 2, using one stage clotting assays. Clot times shown are averages of duplicates. Protein C clot times were determined using the method described by Francis & Seyfert (Am. J. Clin. Path., 87:619-625, 1978). Factor IX was not measured against a buffer clot time because of the extremely high activity in the monoclonal antibody purified product.

EXAMPLE 3

Polyacrylamide Gel Electrophoresis and Western Blotting:

SDS- polyacrylamide gel electrophoresis (SDS-PAGE) was performed by the method of Laemmli (Nature 227:680, 1970) utilizing the Pharmacia Phastgel system and the Hoeffer Mighty Small Vertical Slab (MSVS) Gel Model SE200. In the Heoffer MSVS, a 4% acrylamide stacking gel preceded a 9% acrylamide separating gel. The Pharmacia Phastgel system utilized an 8-25% gradient gel. Proteins were diluted to the appropriate concentration in TRIS-buffered saline 0.1% SDS with or without 2-mercaptoethanol.

Following electrophoresis on SDS gels, a portion of each gel was stained with Coomassie Brilliant Blue R-250, while the proteins on the remaining portion of the gels were electroblotted on the nitrocellulose paper overnight by the standard procedure (Towbin et al, Proc. Natl. Acad. Sci. USA 76:4350-4354, 1979). The electrophoretic transfer was carried out overnight at 20 volts. Following the transfer, membranes were washed in TRIS buffer containing 3% bovine serum albumin and 0.1% Tween 20 for 2 hours as a blocking step. After further washing in buffer with reduced albumin (0.1%), the membranes were cut into strips to be probed with polyclonal rabbit antisera to human FIX (Accurate, lot 014A), prothrombin (Behring, lot 010508), FX (Diagnostic Stago, lot 114) and human protein C (Diagnostica Stago, lot 6006E15). The membranes were incubated with these antibodies for two hours, washed extensively and then incubated with goat-anti-rabbit conjugated to horse radish peroxidase (Biorad. lot 31816). After extensive washing, the strips were subsequently developed with color reagent containing H_2O_2.

In addition to the western blots against other coagulation factors, western blots were performed against inter-a-trypsin inhibitor and against murine IgG to detect any leakage that might occur from the affinity column. The results are shown in Figures 2 and 3.

Figure 2 is an SDS-PAGE gel along with western blots against Factors II, IX, X and Protein C, both in the starting material loaded on the immunoaffinity column as well as the final product. The starting material in this case was eluate from DEAE Sephadex adsorption of cryo-supernatant poor plasma. As the results clearly indicate, the starting material contains all the three contaminants that were tested. However, none of these contaminants, including Factor II or prothrombin, was present in the monoclonal antibody purified product.

A major problem in utilizing immunoaffinity purification techniques has been the leaching of affinity ligand from the column, causing a contamination of the product with the affinity ligand, usually antibody or antibody fragments. Figure 3 clearly shows that there is no leaching of antibody in accordance with the methodology employed in the present invention. This antibody is known to be reactive against mouse IgG as indicated earlier. The product samples used in this assay were from the first preparatory runs on an immunoaffinity column, and there was no leakage in this case. It is well known that the bulk of any ligand leakage occurs within the first few runs on a column. The absence of any detectable murine IgG shows that the adsorption protocol utilized herein, binds the affinity ligand firmly to the substrate matrix. Also, the critical conditions of the ionic strength, pH and compositions of all the buffers utilized in the process including the mild elution conditions employed herein,

does not disrupt the binding between the substrate matrix and the affinity ligand.

EXAMPLE 4

HPLC and Amino Terminal Sequence Analysis

Monoclonal antibody purified FIX product was run on HPLC on a Vydac C_4 Reverse Phase Column with A as 0.1% TFA and B as 0.08% TFA/MECN. Detection was at 220 nm. Figure 4 shows the output from the HPLC run. Approximately 150 pmol of this HPLC purified FIX was subjected to automated Edman degradation. The sample was applied directly from the HPLC fraction to a polybrene treated filter. Twenty cycles of Edman degradation were performed using an Applied Biosystems 477A protein sequencer using the NORMAL-1 program supplied by the manufacturer. Phenylthiohydantoin amino acids were identified using an Applied Biosystems model 120 analyzer. The initial yield was approximately 75%. The yields of the 20 cycles are shown in Table III. The failure to detect PTH amino acids in cycles 7, 8, 15, 17, 18 and 20 is consistent with the presence of gammacarboxy glutamic acid or cysteine at these positions.

TABLE III

Example 4: Amino Terminal Analysis

Cycle No.	Amino Acid (Exp)	Amino Acid (Act)	pmol
1	TYR	TYR	111
2	ASN	ASN	86
3	SER	SER	58
4	GLY	GLY	91
5	LYS	LYS	57
6	LEU	LEU	104
7	---	GAMMA	---
8	---	GAMMA	---
9	PHE	PHE	64
10	VAL	VAL	58
11	GLN	GLN	64
12	GLY	GLY	70
13	ASN	ASN	65
14	LEU	LEU	60
15	---	GAMMA	---
16	ARG	ARG	31
17	---	GAMMA	---
18	---	CYS	---
19	MET	MET	24
20	---	GAMMA	---

Comparison of experimental (Exp) and Actual (Act) N-terminal sequences. The actual N-terminal amino acid sequence was obtained from published data. Gamma refers to Gamma-carboxy glutamic acid.

The evidence presented herein clearly demonstrates that for the first time a FIX product has been obtained which, by all the criteria employed herein, is found to be completely free of detectable amounts of any human plasma protein, unlike any other FIX preparation heretofore known in the art. Furthermore, the pure FIX of the present invention is obtained in high yields and the product, being devoid of any and all known thrombogenic contaminants, is of course, non-thrombogenic

and is safely useful for replacement therapy in treating patients afflicted with haemophilia B; the purity of the product having been demonstrated by four different methods: N-terminal sequence analysis; Laemmli gel electrophoresis, Western Blot analysis and coagulation assays.

A pharmaceutical composition for replacement therapy in accordance with the present invention, comprises therapeutic amount of the pure FIX of the present invention and pharmaceutically acceptable carrier such as physiological saline, non-toxic sterile buffers and the like.

It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

WHAT IS CLAIMED IS:

1. A pure Factor IX product, free of any thrombogenic factor and contaminating proteins.

2. The product of claim 1 with specific activity greater than 170 IU/mg protein.

3. A pharmaceutical composition comprising therapeutically effective amount of the Factor IX of claim 1 for replacement therapy of hemohilia B, and pharmaceutically acceptable carrier.

4. A method of treating patients afflicted with hemophilia B, comprising administering to a patient afflicted with hemophilia B therapeutically effective amount of the Factor IX of claim 1 to alleviate hemophilia B.

5. An immunoadsorbent having binding capacity for at least 50 IU/mg of anti-FIX monoclonal antibodies and dependent on the presence of divalent cations for said binding.

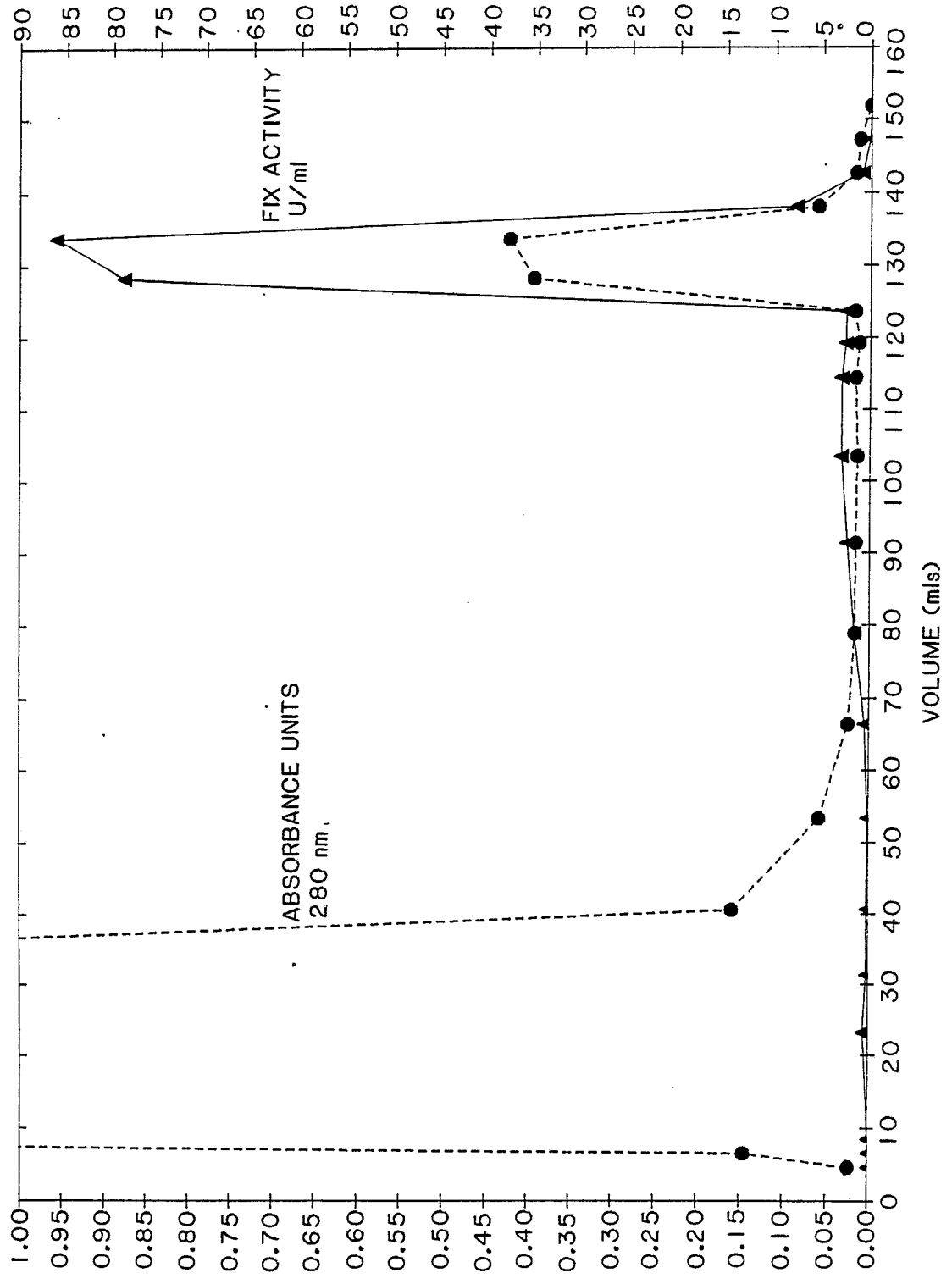

FIX ACTIVITY
U/ml
ABSORBANCE UNITS
280 nm.
VOLUME (mls)
0 5 10 15 20 25 30 35 40 45 50 55 60 65 70 75 80 85 90
0.00 0.05 0.10 0.15 0.20 0.25 0.30 0.35 0.40 0.45 0.50 0.55 0.60 0.65 0.70 0.75 0.80 0.85 0.90 0.95 1.00
0 10 20 30 40 50 60 70 80 90 100 110 120 130 140 150 160

FIG. 1

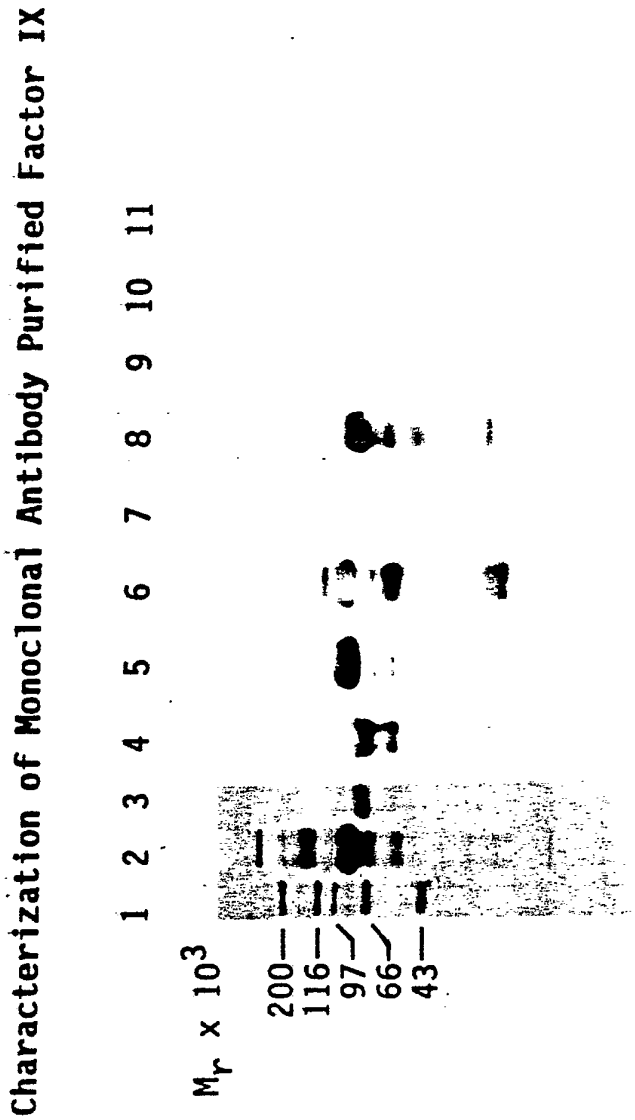
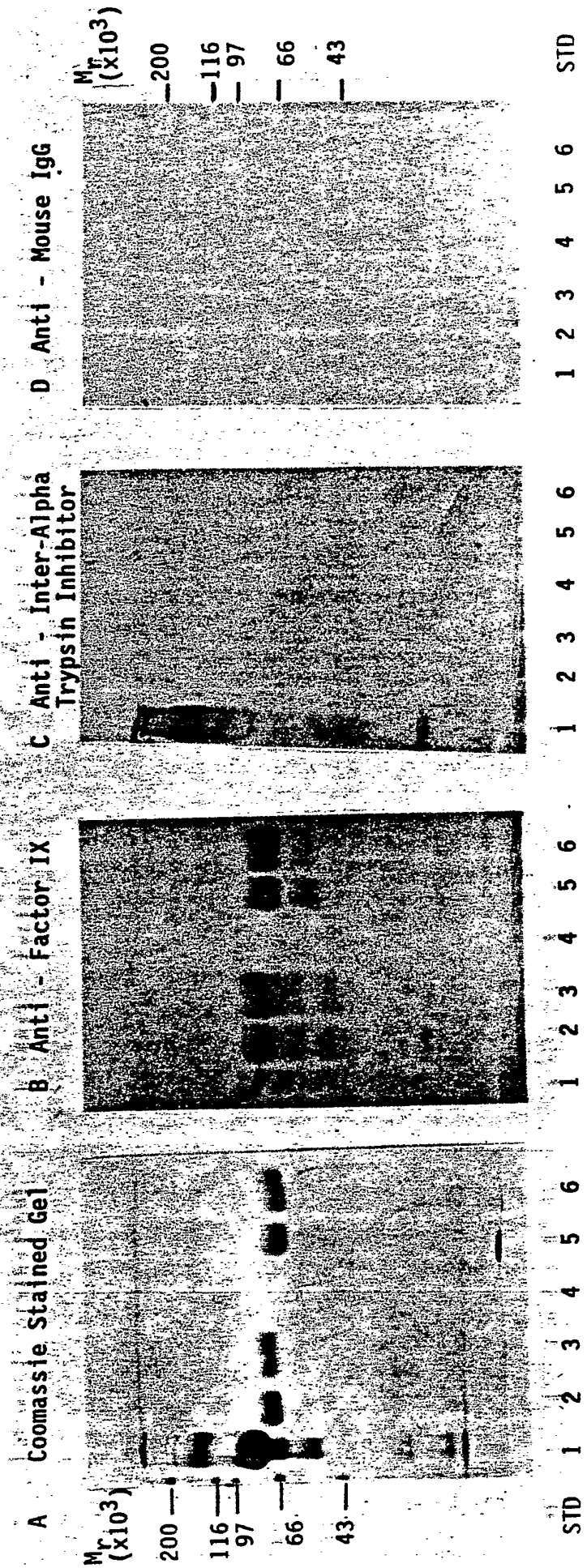

Characterization of Monoclonal Antibody Purified Factor IX
1 2 3 4 5 6 7 8 9 10 11
$M_r \times 10^3$
200
116
97
66
43

FIG. 2


Characterization of Monoclonal Antibody Purified Factor IX
A Coomassie Stained Gel
B Anti - Factor IX
C Anti - Inter-Alpha Trypsin Inhibitor
D Anti - Mouse IgG
M_r ($\times 10^3$)
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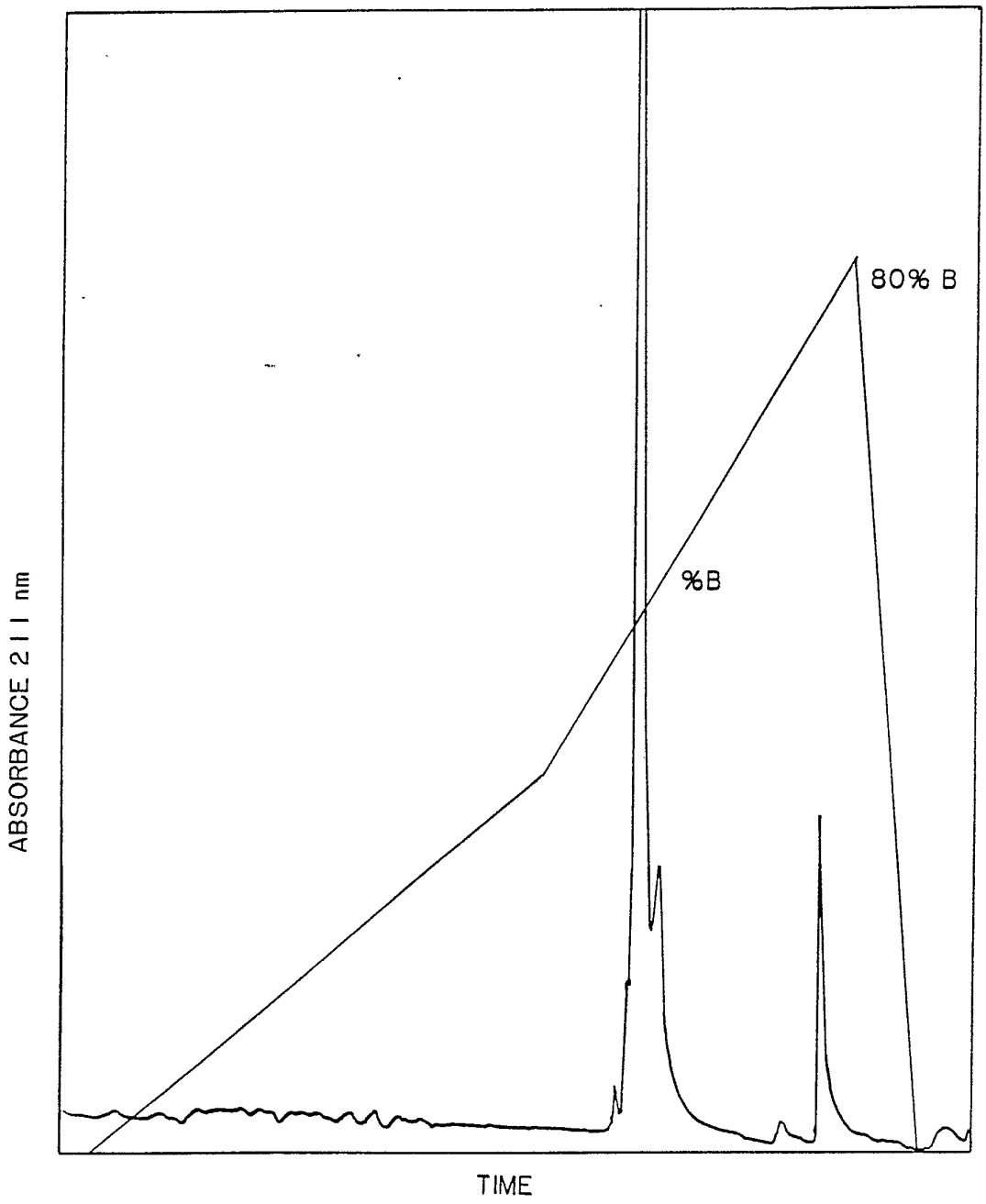
97
66
43
STD
1 2 3 4 5 6

FIG. 3

FIG. 4



INTERNATIONAL SEARCH REPORT

International Application No. PCT/US88/03686

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(4): A61K 37/02;C07K15/14;C07K 15/04;C07K 17/06.
US Cl.: 530/384; 514/8; 530/391; 530/405.

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
US	530/380;530/381;530/384;530/387:530/391;530/405;530/830: 514/8;514/2;514/21;435/172.3;435/68.

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Computer database searches on APS, Biosis.

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category *	Citation of Document, 11 with indication, where appropriate, of the relevant passages 12	Relevant to Claim No. 13
X	Proceedings of the National Acacademy of Science, Vol.82, issued June 1985 (USA), Liebman et al, "Immunoaffinity purification of factor IX (Christmas factor) by using conformation-specific antibodies directed against the factor IX-metal complex," see pages 3879 to 3883.	1-5
X	Biochemistry, Vol.26, No.17, issued 25 August 1987 (USA), Lin et al, "Expression of Human Factor IX and Its Subfragments in Escherichia coli and Generation of Antibodies to the Subfragments," see pages 5267 to 5274.	1-4
X	The Journal of Biological Chemistry, Vol.253, No.17, issued 10 September 1978 (USA), Osterud et al, "Human Blood Coagulation Factor IX," see pages 5946 to 5951.	1-4
X	The Journal of Biological Chemistry, Vol.261, No.21, issued 25 July 1986 (USA), Kaufman et al, "Expression, Purification, and Characterization of Recombinant gamma-Carboxylated Factor IX Synthesized in Chinese Hamster Ovary Cells," see pages 9622 to 9628.	1-5
X,P	US, A, 4,786,726 (SMITH et al), issued 22 November 1988, see columns 2 to 12.	1-5

* Special categories of cited documents: 10

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
16 March 1989	19APR 1989
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Jeff P. Kushan

Form PCT/ISA/210 (second sheet) (Rev. ...)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

Y	Thrombosis Research, Vol.33, issued 1 January 1984 (USA), Smith et al, "Monoclonal antibodies to Factor IX: characterization and use in immunoassays for Factor IX," see pages 211 to 224.	5
Y	US, A, 4,405,603 (SCHWINN et al), issued 20 September 1983, see columns 1 to 4.	1-4

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☐ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.