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COMPARATIVE CHROMATOGRAPHY OF PLASMA AND PLASMA CONCENTRATES RICH IN FACTORS VIII AND IX

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SUMMARY

A method for the comparison of plasma concentrates rich in the coagulation factors VIII (Hemophil™ and Cryoprecipitate) and IX (Konyne™) is presented employing DEAE-cellulose chromatography and immunoelectrophoresis. Whereas complement C-3 and ceruloplasmin was found to be strongly present in the Konyne™ preparation, this was not found to be the case in the factor VIII preparations. Both albumin and IgG were established to be present in all the concentrates; albumin was assigned to the latter parts of the chromatogram whereas IgG was found in all fractions tested. It was again shown that fibrinogen was a contaminant of the factor VIII preparations; Konyne™ was found to be free of fibrinogen in our experiments. Procoagulant activity could be isolated in some of the fractions from the column for the commercial preparations; this was not so for the other samples chromatographed.

INTRODUCTION

The medical treatment of hemophiliac patients has improved very considerably within the past decade. To a certain extent this is due to the purification of antihemophilic globulin (factor VIII) and Christmas factor (factor IX) from human plasma, two proteins which are found in small amounts and are required for normal coagulation of the blood. There are a number of ways in which the purification of these factors has been accomplished. The original attempts to concentrate factor VIII for clinical use were performed by Pohle and Taylor[1]. However, the Cohn fractionation[2], introduced several years later, was really the starting point for modern preparation of various types of clotting factor concentrates. This was followed by modifications of the Cohn technique that yielded material with increased potency as well as containing less protein[3,4]. More recently, a number of intermediate and high-purity factor VIII concentrates have been prepared[5–9]. Even though these methods have improved on the purity of the original preparation of factor VIII, the preparation by simple cryoprecipitation[10] of fresh plasma is still widely used today. In contrast to factor VIII, the purification of factor IX has been to some extent slower to progress with original work accomplished in 1959[11,12], possibly as a result of the lower incidence of

factor IX deficiency compared to factor VIII deficiency. This was followed by the preparations of Biggs *et al.*[13], by Stych and Lechner[14] and by Tullis *et al.*[15]. In all these studies, concentrates were always contaminated with some of the other plasma clotting proteins.

The main purpose of this study was to examine various plasma concentrates of factor VIII and factor IX and to compare these concentrates to whole plasma. Column chromatography on DEAE-cellulose and immunoelectrophoresis were the principal techniques used.

EXPERIMENTAL

Materials

Plasma. Blood was collected into 1/10 volume 0.1 *M* sodium citrate and centrifuged immediately to obtain the plasma.

Serum. Blood was allowed to clot for two hours after which the clots were rimmed and the serum obtained by centrifugation.

Factor VIII concentrates. One type of factor VIII concentrate employed was the antihemophilic globulin (AHG) cryoprecipitate; this material was obtained from the Canadian Red Cross Blood Transfusion Service and is prepared by the method of Pool *et al.*[10]. The serial numbers of these preparations were 0-9643, 0-78277, 0-29686 and 0-29687 and the factor VIII activity ranged from 3–7 units/ml. A second type of factor VIII concentrate studied was Hemophil™ antihemophilic factor (human) obtained from Hyland (division of Travenol Labs., Costa Mesa, Calif., U.S.A.); all the samples that were tested for this concentrate came from the same lot number (lot no.: 0591E87A1); they contained up to 142 units per vial.

Factor IX concentrate. The source of this factor was Konyne™, obtained from Cutter Laboratories (Berkeley, Calif., U.S.A.), lot numbers K5076 and K5813: these contained 500 units per vial. Konyne also contains several other clotting factors (*e.g.* factors II, VII and X).

Methods

Column preparation and elution. The instrumentation employed for chromatographic purposes was the Spectrochrom, Model 130 (Beckman). The evaluation of this instrument has been reported in this journal by Blatt and Pittman[16]. This apparatus allows for the automatic programming, recording and collection of samples at cold temperature. The adsorbent used was DEAE-cellulose (Whatman DE-23, series II, batch no. 2423/56, small ion capacity of 1 mequiv./g), and columns were packed employing 0.04 *M* Tris–phosphate buffer, pH 8.6. The size of the columns was 1.9 × 15 cm and they were jacketed to maintain the temperature at 4°. The amount of material charged to the DEAE-cellulose ranged from 140–280 mg of plasma or concentrate; this was diluted with 2–4 volumes of starting buffer and dialyzed overnight *versus* the starting buffer. The buffer used for the runs was 0.04 *M* Tris–phosphate, pH 4.7. The total volume for a column run was 2 l. In the operation of the runs, 15-ml samples were collected at a flow-rate of 120 ml/h. The chart recorder speed was set at 2 in./h with an absorbance reading set at 280 nm.

Immunoelectrophoresis. The immunoelectrophoretic techniques employed[17] utilized 1% agar (Difco, Detroit, Mich., U.S.A.) and barbital buffer, pH 8.6. Specific

antisera (Hyland) to human plasma protein employed consisted of albumin, ceruloplasmin, complement C-3, fibrinogen, haptoglobin, IgA, IgG, IgM, β-lipoprotein, transferrin and α_2-macroglobulin as well as a multivalent protein antisera. The samples analyzed were obtained from fractions of the various peaks of the chromatograms. They were tested against the specific antisera after dialysis and concentration by lyophilization.

Biologic assays. Assays for factors VIII and IX in selected fractions were tested using the activated partial thromboplastin time[18].

RESULTS AND DISCUSSION

Plasma and serum

Fig. 1 shows typical chromatograms for plasma and serum. The plasma serves as a normal reference for the other types of samples which were chromatographed under identical conditions. Both the plasma and the serum, although not identical, gave rise to three major peaks with shoulders appearing in most. The results of the reactions to specific antisera are shown in Table I. Because of their multiplicity, fractions were chosen which in our opinion may have resulted in a positive arc. The first peak of the plasma (fractions 2–5) indicated, by immunoelectrophoresis, the presence of only IgG, a finding which was duplicated by the serum. The presence of fibrinogen, IgG and transferrin could be detected in the second major peak of the plasma. The same results were obtained in the second peak of the serum except that fibrinogen was not detected; this indicates that all the fibrinogen was lost during the clotting process. The third major peak of both the plasma and serum gave precipitating arcs in agar gel electrophoresis to the specific antisera against human albumin, ceruloplasmin, IgA, IgG, transferrin and α_2-macroglobulin. These findings are in close agreement with the earlier studies of Sober and Peterson[19] and James and Standworth[20] when these investigators used DEAE-cellulose under slightly different conditions; they found

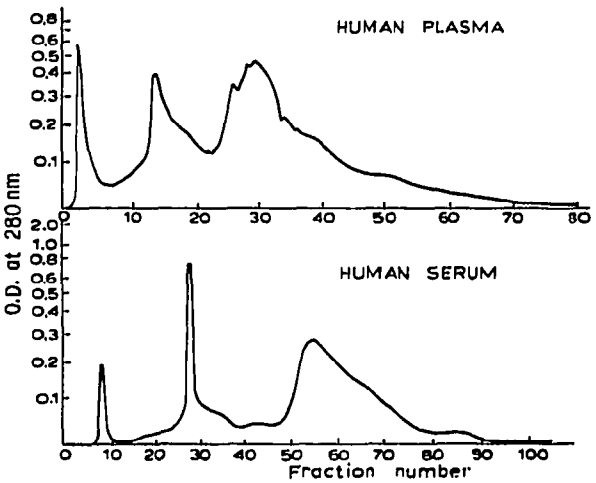

Fig. 1. Chromatogram of human plasma (above) and human serum (below) on DEAE-cellulose. The optical density at 280 nm on a logarithmic scale is plotted *versus* the fraction number.

TABLE I

REACTION TO SPECIFIC ANTISERA

Samples chromatographed	*Fractions tested*	*Albumin*	*Cerulo-plasmin*	*C-3*	*Fibrinogen*	*Haptoglobin*	*α_2-macro-globulin*	*IgA*	*IgG*	*IgM*	*β-lipoprotein*	*Transferrin*
Plasma	2–5	–	–	–	–	–	–	–	+	–	–	–
	12–17	–	–	–	+	–	–	–	+	–	–	+
	27–33	+	+	–	–	–	+	+	+	–	–	+
Serum	7–10	–	–	–	–	–	–	–	+	–	–	–
	16–20	–	–	–	–	–	–	–	+	–	–	–
	25–33	–	–	–	–	–	–	–	+	–	–	+
	39–43	–	–	–	–	–	–	–	+	–	–	+
	49–71	+	+	–	–	–	+	+	+	–	–	+
AHF-Cryoprecipitate	4–7	–	–	–	–	–	–	–	–	–	–	–
	22–28	–	–	–	–	–	–	–	+	–	–	–
	31–37	–	–	–	–	–	–	+	+	–	–	–
	44–53	+	–	+	+	–	+	+	+	–	–	+
Hemofil™-125	6–9	–	–	–	–	–	–	–	+	–	–	–
	22–42	–	–	–	+	–	–	–	+	–	–	–
	72–87	+		–	–	–	–	–	+	–	–	–
Konyne™	10–19	–	–	+	–	+	–	–	+	–	–	–
	34–35	–	+	+	–	–	–	–	+	–	–	–
	39–42	–	–	–	–	–	–	–	+	–	–	–
	71–84	+	–	–	–	–	–	–	+	–	–	–
	109–112	–	+	+	–	–	–	–	+	–	–	–
	137–155	–	–	–	–	–	–	–	–	–	–	–

that both albumin and ceruloplasmin were detected in the latter part of the chromatograms whereas IgG and transferrin also appeared in the earlier peaks.

Cryoprecipitate

When we look at the AHG-cryoprecipitate (Fig. 2 and Table I), the peak containing fraction numbers 22–28 appears to be the same in part as the first peak of the plasma since only IgG can be identified. However, in fractions 44–53, there does appear complement C-3. This is probably the result of the concentration of complement C-3 by the cryoprecipitation procedure. On the other hand, the chromatography of the cryoprecipitate failed to show the presence of ceruloplasmin in any of the tubes tested. Fibrinogen, which has always been a contaminant in attempts to purify antihemophilic globulin, was eluted in the latter fractions when the cryoprecipitate was chromatographed; in the plasma experiments, it was eluted earlier. Shapiro *et al.*[21] have found the latter observation to be true in their recent experiments employing DEAE-cellulose.

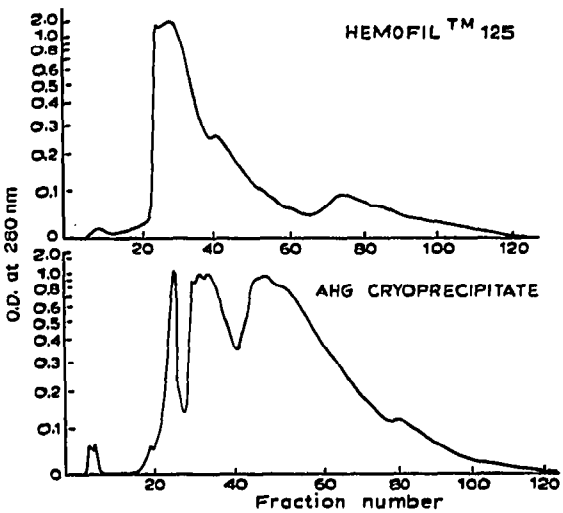

Fig. 2. Chromatogram of Hemophil™ 125 (above) and AHG-cryoprecipitate obtained from the Canadian Red Cross (below) on DEAE-cellulose. The optical density at 280 nm on a logarithmic scale is plotted *versus* the fraction number.

Hemophil™ 125

From the fractions investigated (Fig. 2 and Table I) it can be seen that Hemophil 125 is relatively pure when compared to plasma or the cryoprecipitate by immunoelectrophoresis. As was the case for the cryoprecipitate, fibrinogen is still a contaminant of this type of factor VIII concentrate. As in the other chromatograms, IgG is also consistently identified in all the peaks; albumin is again eluted in the latter part of the chromatograms. Our results on Hemophil™ are in agreement with those found by Johnson's group[8] who prepared a very high purity factor VIII concentrate; these investigators found albumin and fibrinogen in their preparations.

Konyne™

Although the other materials were relatively constant in terms of the peaks, it is interesting to note that chromatography of Konyne™ resulted in chromatograms which were markedly different (Fig. 3). After giving sharp peaks at the beginning (up to about fraction 50), there appeared a very broad peak (fraction 50–200). Besides this, it was found that factor IX concentrate was free of fibrinogen. In the factor VIII

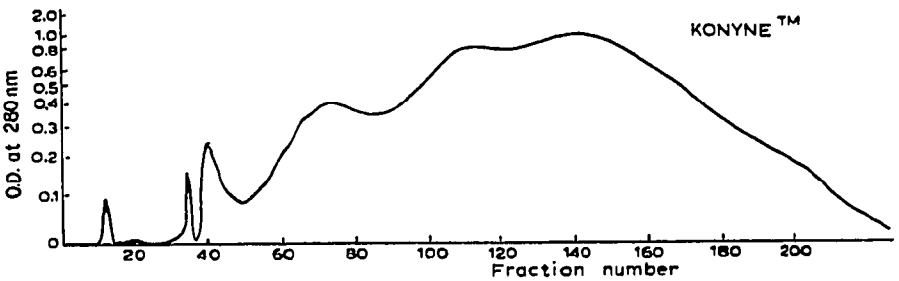

Fig. 3. Chromatogram of Konyne™ on DEAE-cellulose. The optical density at 280 nm on a logarithmic scale is plotted *versus* the fraction number.

concentrates, fibrinogen was always a contaminent; this is important therapeutically because Konyne is used in the treatment of hemophilia B (factor IX deficiency) which is a less frequent type of hemophilia than hemophilia A (factor VIII deficiency). More concentrates are used in the latter case. In contrast to the other materials, it was found that Konyne was a good source of complement and was rich in ceruloplasmin as measured by our immunoelectrophoretic techniques. The chromatography of Konyne did show some similarities to the other materials: albumin was eluted in the same region as the other substances chromatographed and IgG was found to be present in almost all of the fractions tested.

Although consistent results could be obtained by chromatography and immunoelectrophoresis, the assay for either factor VIII or factor IX proved to be disappointing. This perhaps could be explained partly by the procedures used since it is well known that (especially for factor VIII) these proteins are sensitive to isolation procedures. This was found to be the case for the plasma, serum or the cryoprecipitate. However, the commercial concentrates did show significant procoagulant activity. For Hemophil IV, the first fraction resulted in 23% of the applied activity charged to the column whereas the second fraction showed close to 60%. This second fraction was rich in fibrinogen but fraction I was not. When Konyne was used, the greatest factor IX activity was found in the first fraction which contained 40% of the procoagulant activity. The second and third showed 18% and 15% procoagulant activity, respectively; no factor IX activity could be found in the later fractions.

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