The Separation of AHF from Fibrinogen*)

From the Pediatric Clinic of the University of Amsterdam, the Netherlands

S. van Creveld, H. A. Veder, C. N. Pascha and W. F. Kroeze

Introduction

Since long we have been interested in the problem of the purification of the anti-haemophilic factor (1, 2). In a previous article (3) we described the results obtained by the adsorption of human AHF on and its elution from kaolin and bolus alba resp. from plasma fraction I (Cohn [4]). The yield was very small, however, an inconvenience from which also Seegers (5) and Brinkhous (14) have been suffering. In several ways we have tried to concentrate the eluates, i.e. by ultrafiltration in a refrigerated centrifuge according to Peters (6) but, as will be clear, this did not improve the yield and besides the AHF was adsorbed and/or denatured on the collodion membrane.

Because of the affinity of AHF to silicates we also tried to chromatograph it on columns of sand or glasspowder, but the results were unsatisfactory.

Since chromatography of proteins and enzymes has developed rapidly in the past decade we decided to investigate the possibility of purifying the AHF by chromatography on ion-exchange columns. This article gives a survey of the results obtained until now.

Materials and Methods

1. Except when stated otherwise oxalated bovine plasma was used as a source of AHF. Before chromatography the plasma was shaken with $BaSO_4$ (1/10 w/v) for 10 minutes and after centrifuging fraction I was precipitated with alcohol in the cold from the supernatent following Cohn's method. The precipitate was dissolved in the same solvent as used for the chromatographic experiment and besides AHF contained fibrinogen and some alpha-, beta- and gamma-globulins.

*) This work was aided by grants from the Organization for Pure Scientific Research in the Netherlands.

2. Below pH 6.5 acetate-buffers and above pH 6.5 imidazole-buffers were used. In general the solvents were 0.02 M with respect to these buffers.

3. The ion-exchangers used were:

Amberlite IRC 50, 100—200 mesh
Amberlite IR 120, 100—200 mesh
Amberlite IRA 400, 100—200 mesh
Deacidite FF, 52—100 mesh
DEAE-cellulose.*)

4. With the ion-exchange resins columns of different sizes were used varying from 1.2 cm internal ⌀ and 25 cm length to 3.2 cm internal ⌀ and 40 cm length. All cellulose columns had an internal diameter of 1.2 cm and a length of 20—25 cm. The effluent fractions were collected with a Pleuger Universal fraction collector in volumes of 5 cc or, sometimes, on time-basis.

5. In the effluent fractions protein was determined by measuring the light-absorption at 280 mμ in a Unicam Spectrophotometer SP 500, fibrinogen was detected by the clotting action of thrombin and sometimes determined according to Clauss (7). AHF was assayed by its influence on the recalcification time of haemophilic plasma. If necessary the fractions were neutralized on the pH-meter before testing.

Results

The results obtained with ion-exchange resins were disappointing. We tried a great many different salt concentrations, pH's in the range of 5 to 8 and different ions e.g. Li^+, Na^+ and K^+ on the kation-exchangers and Cl^- and acetate$^-$ on the anion-exchangers. In most cases, however, fibrinogen and AHF left the column together without having been adsorbed or even retarded. In some cases, especially in the range of pH 5—6, adsorption (or most probably precipitation) would occur but after changing to a different solvent fibrinogen and AHF again appeared together in the effluent fractions. Only with Deacidite FF (Cl) a small part of the AHF was retained in the column, the greater part leaving it in the presence of fibrinogen. The AHF retained could be eluted by raising the salt concentration. All our attempts to raise the yield of free AHF at the expense of fibrinogen-bound AHF failed.

Most probably our experiments with ion-exchangers would have met with little success if Peterson and Sober (8) had not developed the ion-exchangers on a basis of cellulose, of which especially the diethylaminoethyl-

*) We are obliged to Messrs. Carl Schleicher and Schüll for putting at our disposal part of the DEAE cellulose used in these experiments.

cellulose (DEAE-cellulose) had proved very useful in the separation of serum proteins. As soon as DEAE-cellulose became available we continued our experiments with this product.

To our great disappointment the first experiment was a failure. Again fibrinogen and AHF flowed through the column together, even at the pH which, on Deacidite FF, had proved the most favourable i.e. pH 6.6. Sober, Gutter, Wychoff and Peterson (9) and Fahey, McCoy and Goullian (10) who chromatographed serum on columns of DEAE-cellulose first dialyzed this serum against the dilute buffer with which the experiment was started. We realized that dialysis of plasma against a dilute buffer would inevitably precipitate the AHF and fibrinogen. So we decided to perform the next experiment at as low a salt concentration as possible. To this end the column after aequilibration with buffered salt-solution was washed with water until practically negative Cl-reaction, and the plasma-fraction I was dissolved at the minimum possible salt concentration i.e. 0.4% (physiological saline = 0.9%). This time we found two peaks of fibrinogen both with a NaCl-concentration of 0.37%, but no AHF. Even with 3.5% NaCl it proved impossible to elute the AHF from the column.

The appearance of two fibrinogen-peaks demonstrated the unnecessity of washing the columns with water before use. Before the next experiments they were aequilibrated with 0.4% NaCl and fraction I was dissolved at the same salt concentration. (In later experiments the NaCl-concentration was raised to 0.5%.) This time there was only one peak of fibrinogen but this time too there was no AHF detectable in the effluent fractions.

The protein determinations in the effluent fractions could not account for the total protein brought into the column. The protein retained in the column could be accounted for however, by the amount which left it during its regeneration with 0.5 N NaOH and alcoholic HCl. For this reason we decided to have the regeneration preceded by a gradient elution with 0.3 N NH_4OH containing 1.0% NaCl.

So in the next experiments plasma-fraction I was washed into the column with 0.5% NaCl, buffered at pH 6.6 and after elution of the fibrinogen peak this solvent was gradually replaced by the ammonia solution by means of a mixing chamber. Already at very low ammonia concentration (pH = 8.0) the AHF was eluted from the column in a very sharp peak and with a total recovery of about 100% (Fig. 1).

The same experiments with human plasma fraction I gave a very low yield of AHF which made us look for a different eluent, having no influence on pH. Citrate-, phosphate-, sulfate-, fumarate- and succinate-ions interfere with the

AHF determinations by binding calcium and so will probably do all other di- and tricarboxylic acids. Aromatic acids as well as nitrate-ions have a strong absorption at 280 mμ and in this way interfere with the protein-determinations. Also protein-determinations with Folin-Ciocalteu-reagent could not be performed in the presence of aromatic acids.

Favourable results were obtained with lactic acid (neutralized of course to pH 6.6) as eluent, which was capable to raise the yield of human AHF from a few percent to 25—30% of the amount brought into the column (Fig. 2).

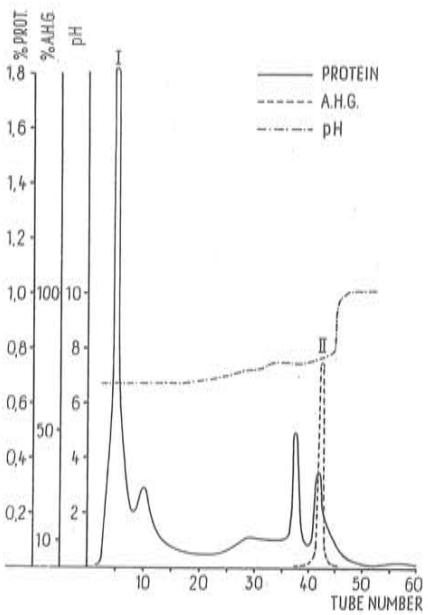

Fig. 1

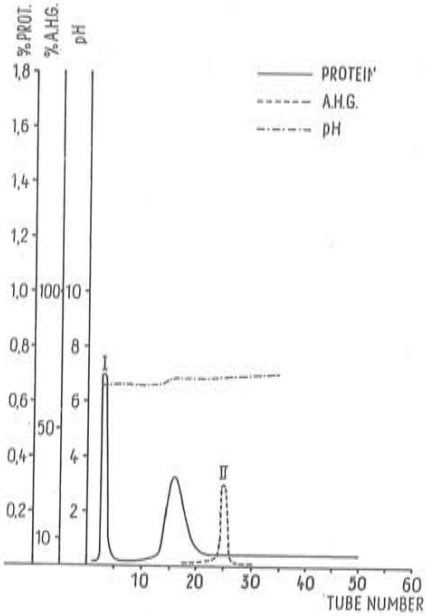

Fig. 2

Fig. 1: Separation of fibrinogen and A.H.G. from bovine plasma fraction I ($BaSO_4$-treated). Solvents:
tube 1— 4: 0.02 M. imidazole-HCl buffer pH 6.6, containing 0.5% NaCl;
tube 5—34: gradient to 0.02 M. imidazole-buffer pH 9.0, containing 1.0% NaCl.
Mixing chamber 200 cc imidazole buffer pH 6.6, 0.5% NaCl.
tube 35—50: gradient to 0.5 M. NH_4OH containing 1.0% NaCl.
tube 50—60: 0.5 M. NaOH without gradient.
Peak I: fibrinogen; Peak II: A.H.G.

Fig. 2: Separation of fibrinogen and A.H.G. from human plasma fraction I ($BaSO_4$-treated). Solvents:
tube 1— 5: 0.02 M. imidazole. HCl-buffer pH 6.6, containing 0.5% NaCl.
tube 6—37: gradient to 1 M. Lactic acid, neutralized to pH 7.0 and containing 0.5% NaCl.
Mixing chamber 200 cc imidazole buffer pH 6.6, 0.5% NaCl.
Peak I: fibrinogen; Peak II: A.H.G.

Ultrafiltration experiments with our AHF preparations showed that AHF did not pass the cellulose-acetate membrane and was concentrated in the upper

fluid. The filtrate did however, contain some other protein which had no influence on the recalcification time of haemophilic plasma.

Discussion

A. From the experiments, mentioned above, it may be concluded, that the complete separation of AHF and fibrinogen, which has been the object of so many investigations (11—17) is easily performed on columns of DEAE-cellulose.

B. The experiments as done on columns of DEAE-cellulose with subnormal salt-concentrations, redone with Deacidite FF gave the same and practically negative result as before on columns of this material.

C. Other conclusions to be drawn from our experiments are:

1. Bovine and human AHF have clearly different properties;
2. The AHF obtainedis not pure and still contains proteins differing from it;
3. The loss, especially of human AHF, is still relatively great. Several factors may be responsible for this fact, e.g.:

a) Partial denaturation of AHF on the surface of the ion exchanger;

b) Oxydation;

c) The affinity of the eluting ions for the ion-exchanger is not great enough to displace the AHF completely;

d) The AHF is not stable enough with regard to the duration of the experiment and the conditions employed.

In accordance with the facts and possibilities just mentioned, we are continuing our investigations on the purification and properties of AHF.

Summary

Anti-haemophilic globulin and fibrinogen of both bovine and human origin could be separated on columns of DEAE-cellulose. The yield of bovine AHF was high in contrast to that of human AHF, but further work to improve this yield is in progress.

Résumé

La globuline antihémophilique et le fibrinogène d'origine bovine et humaine ont pu être séparés par chromatographie sur colonne de DEAE-cellulose. Le rendement de la globuline antihémophilique d'origine bovine est considérable contrairement à celui de la globuline antihémophilique d'origine humaine. Des expériences tendant à améliorer ce rendement sont en cours.

Zusammenfassung

Antihämophiles Globulin und Fibrinogen sowohl von bovinem als auch von menschlichem Plasma konnten mit Hilfe der Säulenchromatographie mit DEAE-Cellulose getrennt werden. Die Ausbeute an bovinem AHF war hoch im Gegensatz zu derjenigen an menschlichem AHF. Weitere Untersuchungen, welche diese Ausbeute zu verbessern trachten, sind im Gange.

References

(1) Creveld, S. van: Sporadische haemophilie. Maandschr. v. Kindergen. 3: 351 (1934).
(2) Bendien, W. M. and Creveld, S. van: Investigations on hemophilia. Amer. J. Diseases Childr. 54: 713 (1937).
(3) Creveld, S. van, Hoorweg, P. G., Ottolander, G. J. H. den, Veder, H. A.: Isolation of the anti-haemophilic factor from human plasma. Acta haematol. 15: 1 (1956).
(4) Cohn, E. J., Strong, L. E., Hughes Jr., W. L., Mulford, D. J., Asworth, J. N., Melin, M. and Taylor, H. L.: Preparation and properties of serum and plasma proteins. IV. A system for the separation into fractions of protein and lipoprotein components of biological tissues and fluids. J. Amer. Chem. Soc. 68: 459 (1946).
(5) Seegers, W. H., Landaburu, R. H., Fenichel, R. L.: Isolation of platelet cofactor I from plasma and some properties of the preparation. Amer. J. Physiol. 190: 1 (1957).
(6) Peters, H. J.: Papierelectrophorese van liquor cerebrospinalis. Chem. Weekbl. 49: 248 (1953).
(7) Clauss, A.: Méthode rapide de détermination du fibrinogène basée sur le temps de coagulation. Acta haematol. 17: 237 (1957).
(8) Peterson, E. A., Sober, H. A.: Chromatography of proteins. I. Cellulose ion-exchange adsorbents. J. Amer. Chem. Soc. 78: 751 (1956).
(9) Sober, H. A., Gutter, F. J., Wijckoff, M. M., Peterson, E. A.: Chromatography of proteins. II. Fractionations of serum protein on anion-exchange cellulose. J. Amer. Chem. Soc. 78: 756 (1956).
(10) Fahey, J. L., McCoy, P. F. and Goulian, M.: Chromatography of serum proteïns in normal and pathologic sera: the distribution of protein-bound carbohydrate and cholesterol, siderophilin, thyroxin-binding protein, B_{12} binding protein, alkaline and acid phosphatases, radioiodinated albumin and myeloma proteins. J. Clin. Invest. 37: 272 (1958).
(11) Lozner, E. L., Kark, R., Taylor, F. H. L.: The coagulation defect in haemophilia: the clot promoting activity in hemophilia of berkefelded normal human plasma free from fibrinogen and prothrombin. J. Clin. Invest. 18: 603 (1939).
(12) Shinowara, G.: Enzyme studies on human blood XI. The isolation and characterization of thromboplastic cell and plasma components. J. Lab. Clin. Med. 38: 11 (1951).
(13) Lorand, L. and Laki, K.: A simple method for purifying an activator of prothrombin. Biochim. biophys. Acta 13: 448 (1954).
(14) Wagner, R. H., Richardson, B. A., Brinkhous, K. M.: A study of the separation of fibrinogen and antihemophilic factor (AHF) in canine, porcine and human plasmas. Thromb. diath. haem. 1: 1 (1957).
(15) Surgenor, D. M., Steele, B. B.: 6th Annual Symposium on blood. January 1957. Thromb. diath. haem. 1: 563 (1957).
(16) Soulier, J. P., Gobbi, F. and Larrieu, M. J.: Séparation du fibrinogène et du facteur antihémophilique A. Rev. d'hématol. 12: 481 (1957).
(17) Blombäck, M.: Purification of antihemophilic globulin I. Some studies on the stability of the antihemophilic globulin activity in fraction I-o and a method for its partial separation from fibrinogen.