Journal of Chromatography, 491 (1989) 299–308
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 4736

CHROMATOGRAPHY OF HUMAN PLASMA ON AMINOHEXYL SEPHAROSE: SEPARATION OF FACTOR VIII/vWf AND BEHAVIOUR OF FACTORS II, VII, IX AND X AND ANTITHROMBIN III

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(First received July 8th, 1988; revised manuscript received February 23rd, 1989)

SUMMARY

Human plasma was chromatographed under different experimental conditions on aminohexyl Sepharose. A strong retention of factor VIII/vWf and of factors VII, II, IX and X was observed. A satisfactory stabilization of eluted factor VIII clotting activity was obtained after addition of albumin and sucrose to the buffers used during chromatography. Albumin and antithrombin III were found apparently intact in the column effluent.

INTRODUCTION

Purifications of the factor (F) VIII/vWf from plasma fractions on AH-Sepharose® were previously reported [1–3]. However, an important activation of the eluted F VIII was observed in all cases. Previous work suggested the addition of sodium citrate, sucrose, albumin and lysine to the elution buffers in order to stabilize F VIII/vWf [4–6].

In this study, we report the results of a systematic investigation for the optimization of adsorption and elution conditions for the purification of non-activated F VIII/vWf from human plasma, using aminohexyl-coupled Sepharose gels. The fate of other plasma components in such purification schemes is examined.

0378-4347/89/$03.50

EXPERIMENTAL

AH-Sepharose was from Pharmacia (Uppsala, Sweden). A second gel (EHD-Sepharose 4B) was prepared by coupling hexamethylenediamine to Sepharose 4B via the oxirane groups after activation with epichlorhydrin, as described previously [7]. The ligand concentration in this case was 225 μmol of hexamethylenediamine per gram of dry gel.

Freshly frozen human plasma from normal subjects, collected on CPD anticoagulant (2.58% sodium citrate, 0.32% citric acid, 0.218% monobasic sodium phosphate and 2.5% dextrose) and human serum albumin were provided by the Regional Blood Transfusion Centre of Lille (CRTS) (Lille, France).

All other chemicals were of analytical grade from Merck (Darmstadt, F.R.G.) or Prolabo (Paris, France).

Chromatographic methods

All chromatographic steps were carried out at room temperature on C 16/40 Pharmacia columns packed with either AH-Sepharose 4B or EHD-Sepharose 4B. The bed volume was 22 ml.

The non-optimized initial chromatographic separations were done by first equilibrating the columns with a 0.1 *M* sodium acetate–0.1 *M* lysine buffer (pH 5.5). Then 220 ml of human plasma were pumped through the columns at 60 ml/h. The columns were washed with the equilibrating buffer until no UV-absorbing material could be detected in the eluent. The desorption was carried out first by adding 0.2 *M* NaCl to the equilibrating buffer and then by adding 0.5 *M* NaCl to the starting buffer and raising the pH to 6.9.

Only AH-Sepharose 4B was chosen for optimization of the buffer composition. This was done by adding 1.0, 3.5 or 10.0% of sucrose and 1.0, 0.5 or 0.1% of human serum albumin to the same buffers as described above. Two columns I and II, were eluted with 0.1 *M* sodium acetate–0.1 *M* lysine (pH 6.9) and 0.5 *M* sodium acetate–0.5 *M* lysine (pH 6.9), respectively. In both the cases, the above-mentioned additives were used to stabilize the F VIII/vWf.

Activity assays

F VIII procoagulant activity (F VIII:C) was measured by a one-stage assay (VIII:C-1) [8] and by a two-stage assay (VIII:C-2) [9]. F VIII antigen (F VIII:Ag) assays were carried out by the solid-phase sandwich technique with alloantibodies from two multitransfused haemophilic patients [10]. Von Willebrand factor antigen (vWf:Ag) was assayed by an electroimmunoassay (EIA) [11] or by an enzyme-linked immunosorbent assay (ELISA) already described [12]. Its ristocetin cofactor activity (vWf:RCo) was assayed by aggregometry using freshly washed platelets [13]. F VIII:C, F VIII:Ag, vWf:Ag and ristocetin cofactor levels were expressed in U/ml, compared with a reference pool plasma calibrated against the First International Reference Prepa-

ration of Factor VIII Related Activities (National Institute for Biological Standard and Control, London, U.K.). Fibrinogen was measured by radial immunodiffusion (Behring plates) and fibronectin was measured by laser nephelometry [14].

Coagulant activities of factors VII, X, IX, II were determined by standard chronometric laboratory tests [15]. Antithrombin III (AT-III) was measured by radial immunodiffusion (AT-III: Ag, Behring plates) and its heparin cofactor activity was determined by a chromogenic method (AT-III: HcO; AT Prest Stago).

The protein content was determined by the Biuret method, and electrophoresis on cellulose acetate and on polyacrylamide gel gradient were performed by the Quality Control and Biochemistry Laboratories of CRTS.

RESULTS

Comparative chromatography of human plasma on EHD-Sepharose 4B and on AH-Sepharose 4B (Pharmacia)

As shown in Table I, F VIII/vWf could be eluted from both columns with the 0.1 *M* sodium acetate–0.1 *M* lysine buffer (pH 6.9), containing 0.5 *M* NaCl. The elution profiles were similar (not shown).

Electrophoresis on cellulose acetate showed that albumin was found intact in the column effluent. Electrophoresis on a gradient polyacrylamide gel (Fig. 1) showed a difference in the protein contents of the fractions eluted from EHD-Sepharose 4B and AH-Sepharose 4B.

A difference between the values for VIII: C-1 and VIII: C-2 was observed for both fractions eluted with 0.5 *M* NaCl from AH-Sepharose 4B and from EHD-Sepharose. There was a notable activation, particularly of F VIII, on EHD-Sepharose 4B.

Optimization of elution conditions for F VIII/vWf on AH-Sepharose 4B in the presence of sucrose and albumin: behaviour of factors II, VII, IX and X and antithrombin III

The results from two sets of three chromatographic assays, each with buffers containing 0.1 *M* sodium acetate–lysine and 0.5 *M* sodium acetate–lysine, with 1% albumin and 1% sucrose as indicated, are shown in Fig. 2 and Table II.

We observed elution of F VIII/vWf in peak 4 with yields of $35 \pm 5\%$ for both elution conditions. F VIII/vWf was more stable when eluted with 1% sucrose and 1% albumin, as shown by the low Δ (F VIII: C-1 and F VIII: C-2) values (0.16 U/ml). This effect was more marked when the 0.5 *M* sodium acetate–lysine buffer was used.

However, the albumin used for stabilization decreased the specific activity of the purified fractions. In order to improve the specific activity, we decreased the albumin concentration to 0.5% while increasing the sucrose concentration

TABLE I

COMPARATIVE CHROMATOGRAPHY OF HUMAN PLASMA ON AH-SEPHAROSE 4B AND EHD-SEPHAROSE 4B

N.D. = not determined.

	Protein (mg/ml)		F VIII:C-1 (U/ml)		F VIII:C-2 (U/ml)		Yield F VIII·C-2 (%)		Specific activity (U/mg)	
	AH-Seph. 4B	EHD-Seph. 4B	AH-Seph. 4B	EHD-Seph. 4B	AH-Seph. 4B	EHD-Seph. 4B	AH-Seph. 4B	EHD-Seph. 4B	AH-Seph. 4B	EHD-Seph. 4B
Plasma:										
Peak 1 (unretarded)	50.5	54.5	0.02	0.06	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Peak 2	5.5	4.5	0.02	0.08	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Peak 3 (+0.2 *M* NaCl)	9	7	0.25	1.20	N.D.	N.D.	N.D.	N.D	N.D.	N.D.
Peak 4 (+0.5 *M* NaCl)	2.1	3.5	1.8	4.00	1.10	0.69	20	9	0.2	0.52

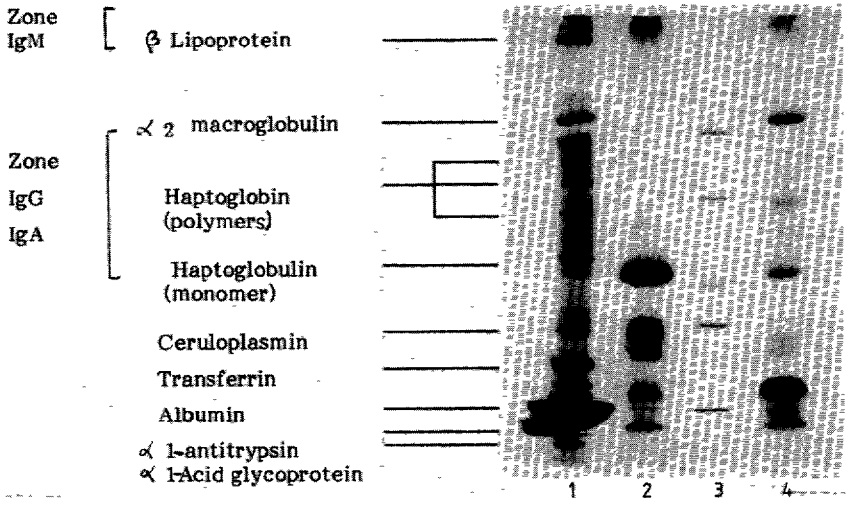

Fig. 1. Comparative electrophoresis on a 4–30% polyacrylamide gel gradient of fractions eluted with 0.5 *M* NaCl from EHD-Sepharose 4B epoxyhexamethylene diamine and from AH-Sepharose 4B. Lane 1, human plasma; lane 2, fraction eluted with 0.5 *M* NaCl on EHD-Sepharose 4B; lane 3, check samples: thyroglobulin (M_r=669 000), ferritin (M_r=440 000), catalase (M_r=232 000), lactate dehydrogenase (M_r=140 000), bovine albumin (M_r=67 000); lane, 4, fraction eluted with 0.5 *M* NaCl from AH-Sepharose 4B.

to 3.5% in both sodium acetate–lysine buffers previously used (Table II and Fig. 2B). As in the previous case, the higher buffer concentration (0.5 *M*) gave better results, with a low level of activation of F VIII:C. However, only a slight decrease in yield was observed. A lower concentration of albumin (0.1%) with a further increase (to 10%) of sucrose in the buffer improved the specific activity. However, in this case due to the partial desorption of F VIII:C at a lower NaCl concentration (0.2 *M*) along with other proteins, the yield of F VIII in peak 4 was significantly lower.

However, the cumulative yields of the eluted peaks, excluding the unretarded major peak of albumin, from the total plasma vary between 40 ± 5 and 60 ± 5% in all cases. It could thus be possible to use a pool of fractions eluted between 0.2 *M* and 0.5 *M* NaCl as material for a second chromatography to improve the specific activity.

Characterization of different plasma components collected during plasma chromatography on AH-Sepharose 4B, using sucrose and albumin

To locate factors II, VII, X and IX and AT-III, during chromatography, the different factors were assayed as described under Experimental.

The results obtained with the different peak fractions from the low molarity (0.1 *M* sodium acetate–lysine) as well as the high molarity (0.5 *M* sodium

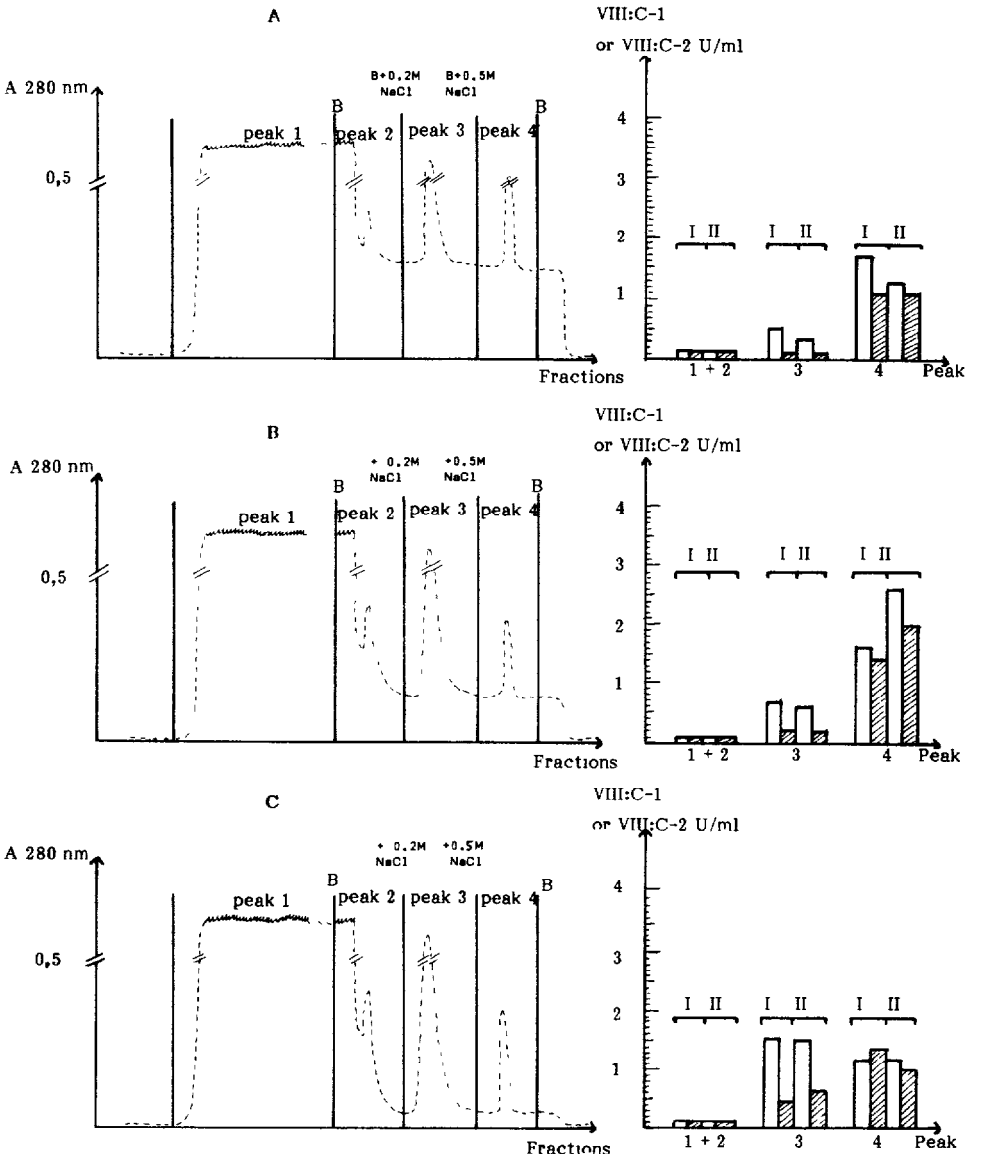

Fig. 2. Chromatographic separations of plasma on AH-Sepharose 4B, carried out during optimization of elution conditions. Also shown are the corresponding histograms for the comparison of VIII·C-1 (white) and VIII·C-2 (shaded) values. (A) Column equilibrated with 0.1 *M* sodium acetate–0.1 *M* lysine–1% sucrose buffer (pH 5.5) (buffer B); elution, peak 3 = B + 1% albumin + 0.2 *M* NaCl; peak 4 = B + 1% albumin + 0.5 *M* NaCl (pH 6.9). (B) Column equilibrated with 0.1 *M* sodium acetate–0.1 *M* lysine–3.5% sucrose buffer (pH 5.5) (buffer C); elution, peak 3 = C + 0.5% albumin + 0.2 *M* NaCl; peak 4 = C + 0.5% albumin + 0.5 *M* NaCl. (C) Column equilibrated with 0.1 *M* sodium acetate–0.1 *M* lysine–10% sucrose buffer (pH 5.5) (buffer D); elution, peak 3 = D + 0.1% albumin + 0.2 *M* NaCl; peak 4 = D + 0.1% albumin + 0.5 *M* NaCl.

TABLE II

COMPARISON OF RESULTS OBTAINED DURING OPTIMIZATION OF ELUTION CONDITIONS ON AH-SEPHAROSE 4B

SA = specific activity; Δ = F VIII:C-1 – F VIII:C-2 = activated F VIII.
(A) Column equilibrated with 0.1 *M* sodium acetate–0.1 *M* lysine–1% sucrose buffer (pH 5.5); 1: elution with 0.1 *M* sodium acetate–0.1 *M* lysine–0.1% sucrose–1% albumin–0.5 *M* NaCl buffer (pH 6.9); 2: elution with 0.5 *M* sodium acetate–0.5 *M* lysine–1% sucrose–1% albumin–0.5 *M* NaCl buffer (pH 6.9).
(B) Column equilibrated with 0.1 *M* sodium acetate–0.1 *M* lysine–3.5% sucrose buffer, (pH 5.5); 1: elution with 0.1 *M* sodium acetate–0.1 *M* lysine–3.5% sucrose–0.5% albumin–0.5 *M* NaCl buffer (pH 6.9); 2: elution with 0.5 *M* sodium acetate–0.5 *M* lysine–3.5% sucrose–0.5% albumin–0.5 *M* NaCl buffer (pH 6.9).
(C) Column equilibrated with 0.1 *M* sodium acetate–0.1 *M* lysine–10% sucrose buffer (pH 5.5); 1: elution with 0.1 *M* sodium acetate–0.1 *M* lysine–10% sucrose–1‰ albumin–0.5 *M* NaCl buffer (pH 6.9); 2: elution with 0.5 *M* sodium acetate–0.5 *M* lysine–10% sucrose–1‰ albumin–0.5 *M* NaCl buffer (pH 6.9).

Buffer system used[a]	A: 1% sucrose + 1% albumin			B: 3.5% sucrose + 0.5% albumin			C: 10% sucrose + 0.1% albumin		
	Δ (U/ml)	Yield (%)	SA (U/mg)	Δ (U/ml)	Yield (%)	SA (U/mg)	Δ (U/ml)	Yield (%)	SA (U/mg)
A	0.59	39	0.71	0.23	27	0.46	0	13.3	0.65
B	0.16	37	0.54	0.55	34	0.82	0.25	11.3	0.5

[a]Buffers: A = 0.1 *M* sodium acetate–0.1 *M* lysine (pH 6.9)–0.5 *M* NaCl; B = 0.5 *M* sodium acetate–0.5 *M* lysine (pH 6.9)–0.5 *M* NaCl.

acetate–lysine) equilibrating buffers containing 0.5% albumin and 3.5% sucrose are summarized in Tables III and IV.

AT-III was found in the column effluent and was apparently unaltered by its passage through the gel since it preserved its biological properties (antigenic and heparin cofactor). Thus it could be easily recovered from the effluent.

Factors IX, VII, X and II are adsorbed on the AH-Sepharose 4B. Factors II, X and IX were eluted with 0.5 *M* NaCl, along with factor VIII. Only factor VII was eluted separately at 0.2 *M* NaCl. It may be possible to separate the factors II, VII, IX and X from F VIII by elution with intermediate steps or a gradient between 0.2 *M* and 0.5 *M* NaCl.

DISCUSSION

The comparative chromatography of plasma on EHD-Sepharose 4B and AH-Sepharose 4B shows a difference in the protein content of the fraction eluted at 0.5 *M* NaCl on the two gels. Electrophoresis of the two eluted fractions on a polyacrylamide gel gradient (Fig. 1) illustrates this difference well. The fraction eluted from EHD-Sepharose 4B exhibits three principal bands, at M_r

TABLE III

COMPLEMENTARITY MEASUREMENTS FOR THE DETECTION OF FACTORS II, VII, IX AND X AND OF ANTITHROMBIN III IN THE DIFFERENT FRACTIONS FROM COLUMN I AND FOR THE FRACTION ELUTED WITH 0.5 *M* NaCl

(A) 0.1 *M* sodium acetate–0.1 *M* lysine–3.5% sucrose–0.5% albumin; (B) 0.5 *M* sodium acetate–0.5 *M* lysine–3.5% sucrose–0.5% albumin. AT-III Ag = antithrombin III antigen; AT-III · Hep Cof = antithrombin III heparin cofactor.

Fraction	Factors				F VIII		AT-III	
	IX (U/100 ml)	VII (U/100 ml)	X (U/100 ml)	II (U/100 ml)	VIII:C-1 (U/100 ml)	VIII C-2 (U/100 ml)	AT-III:Ag (mg/100 ml)	AT-III Hep Cof (U/100 ml)
Column I								
Plasma	82	87	100	100	41	37	25	108
Peak 1 (unretarded)	3	2	5	3	2	–	18.8	102
Peak 2 (A)	5	5	10	5	2	–	0	0
Peak 3 (A+0.2 *M* NaCl)	5	720	20	6.5	67	20	0	0
Peak 4 (A+0.5 *M* NaCl)	760	30	1400	770	160	137	0	0
Column II								
Peak 4 (B+0.5 *M* NaCl)	1000	40	1680	1000	260	205	0	0

TABLE IV

YIELDS AND SPECIFIC ACTIVITIES FOR FACTORS II, VII, IX AND X

CA = clotting activity; SA = specific activity.

Factors and elution conditions	CA (U/ml)	CA (total U)	Yield (%)	SA (U/mg)	
				With albumin	Without albumin
Column I					
IX eluted at 0.5 *M* NaCl	7.6	121.6	67	0.85	2.5
VII eluted at 0.2 *M* NaCl	7.2	241.2	100	0.85	2.9
X eluted at 0.5 *M* NaCl	14	224	100	1.55	4.66
II eluted at 0.5 *M* NaCl	7.70	123.2	56	0.85	2.6
Column II					
IX eluted at 0.5 *M* NaCl	10	135	75	1.18	4
X eluted at 0.5 *M* NaCl	16.8	227	100	2	6.7
II eluted at 0.5 *M* NaCl	10	135	61	1.18	4

67 000, 140 000 and 232 000 (α-1-antitrypsin and albumin, transferrin and ceruloplasmin, monomeric haptoglobin). On AH-Sepharose 4B, electrophoresis reveals the presence of five bands, at M_r 67 000, 140 000, 232 000, 440 000 and 669 000 [α-1-antitrypsin and albumin, transferrin (traces), monomeric haptoglobin, a haptoglobin polymer and α-2-macroglobulin].

The stability of eluted F VIII is higher on AH-Sepharose 4B, the protein environment of F VIII in the fraction eluted at 0.5 *M* NaCl thus appears to have a direct influence on the stability. In fact, we observed the presence of traces of α-2-macroglobulin and haptoglobin polymer, and a higher concentration of albumin in this fraction.

This work showed that AH-Sepharose columns were useful for an enrichment of F VIII/vWf from human plasma, but a complete purification could not be achieved.

The increase in the F VIII/vWf concentration in the eluted fraction and the low level of fibrinogen, previously described by Austen and Smith [2] were reproduced in our system. The low yield of F VIII after elution, which does not exceed 40%, remains the major drawback of this method.

A study of the conditions of elution of F VIII/vWf on AH-Sepharose 4B shows that it is possible, by regulating the relative concentrations of sucrose and albumin added in the buffers during chromatography, to suppress F VIII activation during is elution by 0.5 *M* NaCl and to increase the specific activity (Fig. 2, histogram C, peak 4 and Table II). From batch tests with cryoprecipitate, Faure et al. [6] suggested that sucrose helps to stabilize F VIII/vWf by specifically inhibiting the formation of the complex between F VIII bound to

lectin receptors on the surface of platelet and activated F IX/F X. Our observations concerning the chromatography of plasma in the presence of increasing concentrations of sucrose are in agreement with this hypothesis. F VIII appears remarkably stable when eluted in the presence of 10% sucrose.

CONCLUSION

The use of sucrose and albumin as additives in the chromatographic separation of F VIII:C from plasma on AH-Sepharose 4B seems interesting in terms of the stability of the purified F VIII:C. Moreover, the characterization of other components present in the different fractions after chromatography of plasma on AH-Sepharose 4B (Table III) showed that AT-III is not retained by the gel, but is found in the non-adsorbed material, apparently intact, having retained its biological properties. On the other hand, factors II, VII, IX and X are adsorbed on AH-Sepharose 4B. Factors IX, X and II are eluted with 0.5 *M* NaCl, with F VIII. Only F VIII is eluted separately with 0.2 *M* NaCl. Elution with intermediate concentrations between 0.2 *M* and 0.5 *M* NaCl should allow separate elution of factors II, IX, and X and of F VIII/vWf.

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