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Note

Improved buffer for the chromatographic separation of Factor VIII coagulant

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The activity of activated Factor VIII coagulant first increases and then significantly decreases[1–3]. Although the nature of this inactivation is not clear, a proteolysis mechanism is probably not always involved[4]. The half-live of the molecule depends on the conservation of the whole carbohydrate structure[5–7].

The addition of sodium citrate[7], dextrose and albumin[8] and lysine[9] to the buffers for preparations of therapeutic interest has been suggested. In this study we attempted to explore the possible stabilization of the activity of Factor VIII by different substances during chromatography by the method of Austen[9].

EXPERIMENTAL

All reagents were of analytical-reagent grade.

Fresh human plasma was obtained from normal subjects and contained CPD anticoagulant (2.58% sodium citrate, 0.32% citric acid, 0.218% monobasic sodium phosphate and 2.5% dextrose). Cellular elements were removed by centrifugation and the plasma was chromatographed within 4 h. The cryoprecipitate was adsorbed on aluminium hydroxide. Chromatography was performed on AH-Sepharose (Pharmacia).

Controls performed on the eluates

Protein content was determined by spectrophotometry. Electrophoresis and immunoelectrophoresis were performed in barbiturate buffer (pH 8.6) and stained with amido black. The activity of Factor VIII coagulant was measured in a one-stage assay according to the instructions of the manufacturer (Stago).

Chromatography on AH-Sepharose

AH-Sepharose columns (bed volume *ca.* 30 ml, Pharmacia C 16 column) were pre-equilibrated in the buffer tested at room temperature and plasma or cryoprecip-

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TABLE I

BUFFERS TESTED

Buffer (pH 5.5)	*Components*	*Procedure*	*Molarity*			*Resistivity (mS)*
			Acetate, citrate, histidine	*Lysine*	*NaCl*	
A	Sodium acetate Lysine	Equilibration Elution	0.1 0.1 0.1	0.1 0.1 0.1	 0.2 0.5	9
F	Sodium citrate Lysine	Equilibration Elution	0.01 0.05 0.1	0.01 0.05 0.1		2.5 8 15
G	Histidine Lysine	Equilibration	0.1	0.1		7.5

itate was applied. A constant flow-rate was maintained. Unbound material was removed by washing with the buffer. The elution was obtained with the same buffer containing 0.2 *M* and then 0.5 *M* sodium chloride.

Screening tests

Different amounts (1–1.8 g) of swollen gel equilibrated with the buffer tested were added to 1.5 ml of cryoprecipitate. After incubation and centrifugation, controls were performed on supernatants.

Buffers tested. The buffers were as follows (see Table I):

(A) 0.1 *M* sodium acetate–0.1 *M* lysine, pH 5.5 (ref. 9), 6.1 or 6.5;

(B) buffer A + 10% saccharose;

(C) buffer A + 3.5% saccharose;

(D) buffer A + 1% saccharose;

(E) buffer D + 1% albumin;

(F) 0.01 *M* sodium citrate–0.01 *M* lysine, pH 5.5, 6.1 or 6.5; elution was performed with 0.05 *M* sodium citrate–0.05 *M* lysine and with 0.1 *M* sodium citrate–0.1 *M* lysine;

(G) 0.1 *M* histidine–0.1 *M* lysine, pH 5.5.

RESULTS

As shown in Table II, the screening tests permitted the activity adsorbed per gram of gel. to be evaluated. The best results were obtained with the acetate–lysine buffer. The selectivity of the adsorption of Factor VIII coagulant at pH 5.5 was confirmed by the assays performed with plasma or cryoprecipitate (Tables III and IV). Subsequently the effect of different substances on the recovery of Factor VIII coagulant was studied in acetate buffer (pH 5.5). The results obtained (Fig. 1) demonstrate the effectiveness of buffer E containing saccharose and albumin in the purification of active Factor VIII.

TABLE II

RESULTS OF SCREENING TESTS

Buffer	*Concentration (M)*	*pH*	*Resistivity (mS)*	*Weight of gel (g)*	*Proteins (g/l)*	*Activity of Factor VIII adsorbed (i.u./g of gel)*
A	0.01	5.5	1.4	1.80	6.8	6.650
	0.05	5.5	5	1.80	9.4	6.645
	0.1	5.5	9	1.80	10.2	6.656
	0.1	6.1	9	1.80	9.4	6.653
	0.1	6.5	9	1.80	8.4	6.655
F	0.01	5.5	2.8	1.60	9.2	5.90
	0.05	5.5	8	1.60	11	5.96
	0.1	5.5	15	1.62	13.4	4.70
	0.1	6.1	15	1.60	12.6	2.53
	0.1	6.5	15	1.60	12.6	4.63
G	0.01	5.5	1.5	1.27	7.4	6.27
	0.05	5.5	5	1.44	8.4	6.08
	0.1	5.5	7.5	1.37	10.6	6.40
	0.1	6.1	6.5	1.38	10.6	6.36
	0.1	6.5	6	1.44	10.2	6.07

DISCUSSION

Purification of Factor VIII by chromatography has been applied since 1959 (ref. 10). Although anion exchangers allowed considerable purification, the purified products were unstable. Work was then focused on the stabilization of the molecule[7–9] and on ion exchangers that did not affect the conformation of Factor VIII. The use of aminohexyl-agarose beads at low pH allows the elution of Factor VIII coagulant with an acceptable concentration of sodium chloride[9]. This exchanger also has the advantage of a more rapid equilibrium rate than DEAE-Sephadex[11]. This

TABLE III

CHROMATOGRAPHY OF CRYOPRECIPITATE ON AH-SEPHAROSE

Component	*Volume (ml)*	*Proteins (g/l)*	*Total proteins (g)*	*Factor VIII coagulant (i.u./ml)*	*Factor VIII coagulant (i.u./mg protein)*	*Total activity of Factor VIII coagulant (i.u.)*	*Yield (%)*
Sample	200	8	1.6	1.94	0.24	388	100
Non-adsorbed	240	5.7	1.36	0.07	0.01	16.5	4.25
Buffer A + 0.2 *M* NaCl	70	2.1	0.15	0.2	0.09	14.3	3.7
Buffer A + 0.5 *M* NaCl	155	0.5	0.08	0.93	1.86	155	34
Total						185.8	47

TABLE IV

CHROMATOGRAPHY OF PLASMA ON AH-SEPHAROSE

Component	*Volume (ml)*	*Proteins (g/l)*	*Total proteins (g)*	*Factor VIII coagulant (i.u./ml)*	*Factor VIII coagulant (i.u./mg protein)*	*Total activity of Factor VIII coagulant (i.u.)*	*Yield (%)*
Sample	224	56	12.5	0.59	0.01	132	100
Non-adsorbed	370	31.5	11.65	0.045	0.004	16.5	12.5
Buffer A + 0.2 *M* NaCl	121	3.6	0.44	0.04	0.01	4.8	3.6
Buffer A + 0.5 *M* NaCl	100	1.1	0.11	0.46	0.41	46	34.8

property assumes a gain of time during the process. However, a large part of the coagulant activity of Factor VIII is lost during the purification. This work deals with the protection of Factor VIII during the process.

Three hypotheses were proposed to explain the loss of Factor VIII coagulant activity. First, the action of proteolytic enzymes[1-4] can activate the molecule. Heparin (2 units/ml) and lysine (0.1 *M*) are added to the buffers to overcome this effect. Second, Factor VIII may adsorb to the surface of the exchanger. To eliminate this non-specific adsorption, albumin (1 %, w/v) was added to the buffers. Third, Factor VIII is known to bind to lectinic receptors on the surface of the platelets[12-14] and to form a molecular complex with Factor IX in its activated form and with Factor X[12]. This complex leads to activation and inactivation of the molecule, particularly in the presence of trace amounts of thrombin[15]. The stabilization of Factor VIII coagulant by saccharose[16] may be explained by specific inhibition of the formation of this complex. However, stabilizing effect of high osmolarities is well documented and saccharose has already been used during the heating of Factor VIII coagulant at 60°C for 10 h[17]. Hence we conclude that the so-called non-specific adsorption on insoluble

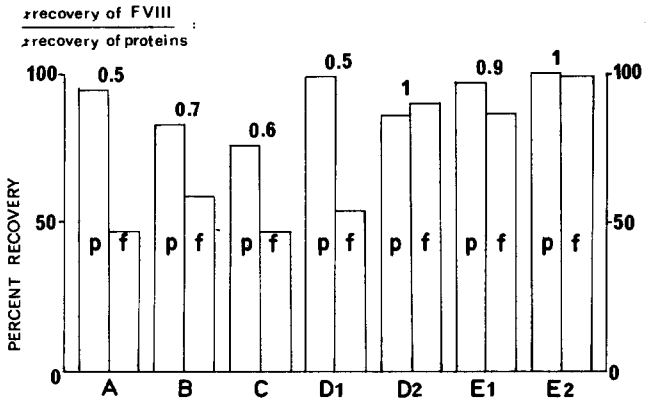

Fig. 1. Recovery of proteins (p) or Factor VIII (FVIII) coagulant (f) by the chromatographic procedure. A–E refer to the buffer used. In all instances except D2, where plasma was chromatographed, cryoconcentrate was used. E1 and E2 refer to different experiments with the same buffer.

polymers with spacer arms is suitable, using appropriate buffers, as a separation tool for blood plasma fractionation.

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