Application of Macroporous Glucomannan Hard Gel to High Performance Liquid Chromatographic Preparation of Coagulation Factor VIII

Masato YAMAGUCHI,* Tetsuro YAMAMOTO,† Takashi TOMODA, and Yoshiaki MOTOZATO††
Kurita Water Industries Ltd., 4-7, Nishi-shinjuku 3-chome, Shinjuku-ku, Tokyo 160
† Department of Molecular Pathology, Graduate School of Medical Sciences, Kumamoto University, 2-2-1 Honjo, Kumamoto 860
†† Department of Industrial Chemistry, Kumamoto Institute of Technology, Ikeda, Kumamoto 860
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Glucomannan spherical hard gels with macroporous nature were 2-diethylaminoethylated (DEAE–) and evaluated for the applicability to preparation of blood coagulation factor VIII.

Adsorption capacity measured by a batch-wise method, of various kinds of DEAE-spherical gels to factor VIII in plasma cryoprecipitate fraction was apparently dependent on Mlim of the gels. DEAE-GM gel (Mlim 1×10^7) possessed the capacity at least 2 to 3 fold higher than those of EMD DEAE-650®, DEAE-Toyopearl 650® and DEAE-Cellulofine A-800®. DEAE-GM gel (Mlim 1×10^7) was applied to high performance liquid chromatographic (HPLC) preparation of factor VIII from plasma cryoprecipitate fraction under high flow rates as rapid as 1 to 4 m h^{-1} in linear velocity. DEAE-GM gel columns in HPLC possessed the high adsorption capacity (30 to 60 U/ml-gel) and good elution recovery from column (51—83%) accompanied by the resulting specific activity as good as 22-113 U/mg-protein. DEAE-GM gel must be a suitable tool in column chromatographic separation of factor VIII and other large proteins in manufacture scale.

Recently, hard and hydrophilic spherical gels have been prepared from a natural glucomannan material. The glucomannan gels are highly porous and the exclusion limit (Mlim) is adjustable from 10^2 to over than 10^7 as measured with polyethylene glycol, keeping the physicochemical stability.[1] Because of the characteristics, glucomannan spherical gels seem to be suitable as gel matrix in column chromatographic preparation of macromolecules not only in low pressure conventional method but also in the high pressure liquid chromatography (HPLC). In the history of HPLC, applicable area of the method has been expanded from smallest size organic molecules to larger molecules, and the expansion seems to depend mainly upon development of gel matrices.

In the present study, we prepared 2-diethylaminoethylated glucomannan (DEAE-GM) gels, and attempted to evaluate the gels for the applicability to HPLC separation of large molecular size proteins. In the research process, we found out that DEAE-GM gels were beneficial in preparation of largest size with the exchange capacity higher than conventional gel matrices. This information suggested the applicability of the gels to preparation of blood coagulation factor VIII. Whereas factor VIII is a plasma protein with the molecular size 280000, it is presented in plasma as oligomeric complexes with von Willebrand factor having the molecular weight as large as 1×10^6 to 2×10^7.[2] Therefore, for preparation of the complexes in HPLC, spherical gel matrices with macropores in which such large molecules can enter, and with the physico-chemical gel stability strong enough in HPLC are required. On the other hand, blood coagulation factor VIII concentrate is an essential requirement for treatment of hemophilia A. For the manufacture scale preparation of factor VIII in the concentrates, it is demanded to improve the yield and to increase the purity of it, in order to achieve self-sufficiency in more countries and to reduce the risk of transmissible diseases for the patients. In this situation development of HPLC columns applicable for the manufacture scale preparation of factor VIII in future must be an important research project. We presently demonstrate that DEAE-GM gel is a good candidate as the gel matrix being required.

Experimental

Materials. Crude glucomannan from amorphophallus konjac was obtained from Shimizu Chemical Co. (Hiroshima, Japan). Citrated human plasma which was normal except for possessing slight chylomicronemia was purchased from a regional branch of the Japan Red Cross Blood Transfusion Service. A manufactory preparation of plasma cryoprecipitate fraction was supplied by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). Bovine serum albumin, α-lactoalbumin, ferritin and thyroglobulin were purchased from Sigma (St. Louis, MD). Factor VIII assay kit, Testzyme factor VIII® was a product of Kabi Vitrum AB (Stockholm, Sweden) and factor VIII standard plasma was a product of Dade (Miami, Florida). Factor VIII deficient plasma was obtained from George King Biomedical Inc. (Overland Park, KA). DEAE-Sepharose CL-6B was a product of Pharmacia (Uppsala, Sweden). DEAE-Toyopearl 650(S) was a product of Tosoh (Tokyo, Japan) and EMD DEAE-650(M) was a product of E. Merck (Darmstadt, Germany). DEAE-Cellulofine A-800 was a product of Chisso (Tokyo, Japan). Polysulfon 0.45 μm pore size filter was a product of Kurabo (Osaka, Japan).

All other chemicals were obtained from Wako Pure Chemicals (Osaka, Japan) and Nacalai Chemicals (Kyoto, Japan).

Preparation of Glucomannan Spheres and the Diethylaminoethylation. After purification with ethanol, glucomannan was sphered and macroreticulated by the method previously described.[1] Ultramicro morphology of the product observed under scanning electron microscopic

examination is shown in Fig. 1. Five types of glucomannan spheres which had the exclusion limit as measured with polyethylene glycol, 4.4×10^5 (GM 440), 7×10^5 (GM 700), 3×10^6 (GM 3000), 6×10^6 (GM 6000), and 1×10^7 (GM 10000) and the sizes in diameter, 20 to 44 μm, 32 to 90 μm, 20 to 44 μm, 20 to 44 μm and 32 to 90 μm, respectively, were prepared.

Properties of DEAE-matrices are shown in Table 1 and the representative calibration curves of them are shown in Fig. 2.

Preparation of Cryoprecipitate Fraction. Defibrinogenated cryoprecipitate fraction of human plasma was prepared according to the method of Thorell and Blombäck[3] with a modification by the use of protease inhibitors as follows. During the preparation except for the final stock solution of cryoprecipitate, protease inhibitors such as soybean trypsin inhibitor, benzamidine and diisopropyl fluorophosphate were added at the final concentrations 4 μM, 12.5 mM, and 1 mM, respectively. Specific activity of factor VIII in the cryoprecipitate fraction was from 0.8 to 1.1 U/mg-protein. The defibrinogenated cryoprecipitate stock solution in 55 mM citrate buffer (pH 7.4) was stores at −70°C until use.

Crude cryoprecipitate prepared by the Chemo-Sero-Therapeutic Research Institute in a conventional way without defibrinogenation, and stored at −70°C in blocks was dissolved into 55 mM citrate buffer (pH 7.4) and passed through the 0.45 μm pore size filter. The soluble fraction of crude cryoprecipitate was freshly prepared at each experiment. The specific activity of factor VIII was from 0.13 to 0.24 U/mg-protein.

Assay of Factor VIII. Two different functional assays were used. One was to measure the correction capacity for the coagulation time prolonged in factor VIII deficient plasma (clotting assay) using a coagulometer (Amelung Coagulometer KC1A). The other was to measure the acceleration capacity of factor VIII in factor Xa generation from factor X by factor IXa,[4] using the enzymatic assay kit, Testzyme factor VIII®. In this assay, an end point measurement was carried out according to the method recommended by the company. Due to the accuracy and reproducibility, the latter assay was mainly used. To make titration curve, serially diluted standard plasma was utilized and the activ-

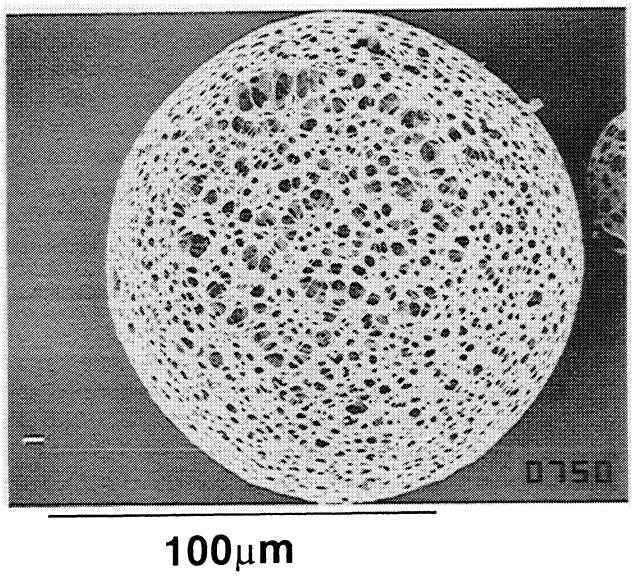

Fig. 1. A scanning electron microscopic (SEM) picture of glucomannan gel.

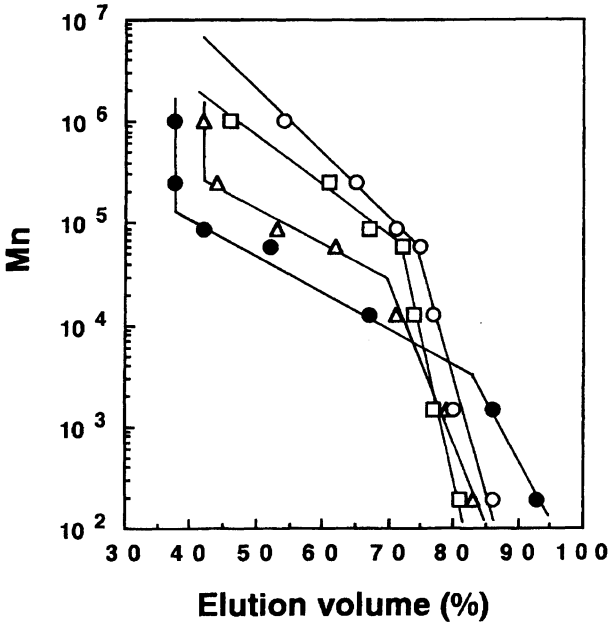

Fig. 2. Representative calibration curves of DEAE-matrices. The elution volume is expressed as a percentage of the column volume (100 Ve/Vt). (○) DEAE-GM 10000, (□) DEAE-GEM 3000, (△) DEAE-Toyopearl 650(s), (●) DEAE-Cellulofine A-800. HPLC conditions: Pump: Shimadzu LC-6A, Column size: 6 mm×100 mm, Flow rate: 2 m h^{-1}, Detector: RI, STD: Polyethylene glycol.

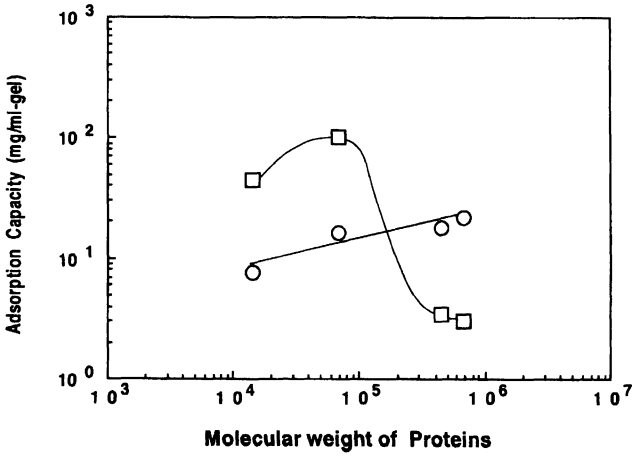

Fig. 3. Correlation between adsorption capacity of DEAE-matrices and molecular weight (M_w) of proteins. (○) DEAE-GM 10000, (□) DEAE-Sepharose CL-6B. Samples: 1. α-Lactalbumin (M_w: 1.4×10^4) 2. Bovine Serum Albumin (M_w: 6.7×10^4) 3. Ferritin (M_w: 4.4×10^5) 4. Thyroglobulin (M_w: 6.7×10^5) HPLC conditions: Pump: Shimadzu LC-6A, Column size: 3 mm×40 mm, Detector: UV, Wavelength: 280 nm.

Table 1. Properties of DEAE-Matrices

No.	Name	Particle diameter μm	Exclusion limit[b] (as PEG)	Maker	Ion-exchange capacity(meq/g-gel)
1	DEAE-GM 440	20—44	4×10^5	Kurita	1.2
2	DEAE-GM 700	32—90	7×10^5	Kurita	1.4
3	DEAE-GM 3000	20—44	3×10^6	Kurita	1.1
4	DEAE-GM 6000	20—44	6×10^6	Kurita	1.2
5	DEAE-GM 10000	32—90	1×10^7	Kurita	1.2
6[a]	DEAE-Toyopearl 650(S)	25—44	3×10^5	Tosoh	0.8[c]
7[a]	EMD DEAE-650(M)	44—88	3×10^5	E. Merck	—[c]
8[a]	DEAE-Cellulofine A-800	53—125	1×10^5	Chisso	0.8[c]

a) Commercially obtained. b) Presently measured. c) Announcement by the companies.

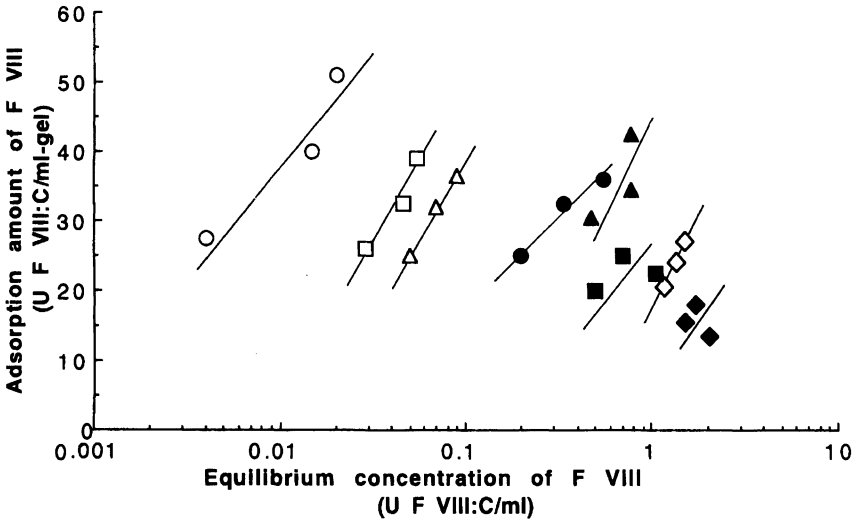

Fig. 4. Adsorption isotherms of batch-wise adsorption of factor VIII on various DEAE-matrices. Experiments were carried out at 20°C for 1h using cryoprecipitate solutioin (citrate buffer, pH 7.4). (○) DEAE-GM 10000, (□) DEAE-GM 6000, (△) DEAE-GM 3000, (●) DEAE-GM 700, (■) DEAE-GM 440, (▲) EMD DEAE-650(M), (◇) DEAE-Toyopearl 650(S), (◆) DEAE-Cellulofine A-800. Cryoprecipitate solution: F VIII: 1.98 U ml^{-1}, Proteins: 1.80 mg ml^{-1}, Specific activity: 1.10 U/mg-protein.

ity in samples was expressed as units of factor VIII.[5] One unit was defined as the amount of factor VIII in one ml of the standard plasma.

Adsorption of Factor VIII to DEAE-Spherical Gels by Batch-Wise Method. The gel beads(50 μl) in plastic tubes were initially washed with 20 mM Tris-HCl buffer (pH 7.4) containing 1 M NaCl, then equilibrated with 20 mM Tris-HCl buffer (pH 7.4). 700, 900, and 1100 μl aliquots of the defibrinogenated cryoprecipitate in 55 mM citrate buffer (pH 7.4) were added into the tubes, respectively, and incubated for 60 min with an end-over-end rotator at 20°C. The mixtures were then centrifuged for 3 min at 10000 rpm with a micro centrifuge (Kubota, Japan) at 20°C, and the factor VIII contents in the supernatants were measured as described above. In the calculation of adsorption capacity, free water space in the gel beads was neglected because of the smallness.

Column Chromatography of Factor VIII. A DEAE-GM 10000 gel slurry suspended in 20 mM Tris-HCl buffer (pH7.4) containing 100 mM NaCl was packed into stainless steel columns (6 mm ×100 mm, bed volume 2.83 ml) with an HPLC system (Shimadzu LC-6A). The final flow rate at the packing was 10 m h^{-1} in linear velocity. The DEAE-GM 10,000 column was equilibrated with 20 mM Tris-HCl buffer (pH 7.4) and the crude cryoprecipitate solutions in 55 mM citrate buffer (pH 7.4) was applied to the column at the flow rate 1—2 m h^{-1}, then eluted step-wisely at the flow rate 4 m h^{-1} with 20 mM Tris-HCl buffer (pH 7.4) containing various concentrations of NaCl as described below. The HPLC was carried out at 20°C by the use of a LKB-system with a Super-loop® sample applicator.

Results and Discussion

Adsorption Capacity Profile of DEAE-Spherical Gels Regarding to Molecular Size of Proteins Adsorbed. Adsorption capacities to α-lactalbumin (M_w 14000), bovine serum albumin (M_w 67000), ferritin (M_w 440000), and thyroglobulin (M_w 670000) of DEAE-GM 10000 and DEAE-Sphearose CL-6B were measured. As shown in Fig. 3, adsorption capacity of the former was almost constant to these proteins, while that of the latter depended upon the molecular size of the sample and dramatically changed between bovine serum albu-

Table 2. Analytical Data for Factor VIII in the HPLC Fractions with DEAE-GM 10000 Column

Fraction	Elution buffer[a)]	Recovery of F VIII		Recovery of proteins		Specific activity (U/mg-protein)
		Amount(U)	Yield(%)	Amount(mg)	Yield(%)	Each fraction
1[b)]	(A)	37.84	21.4	828.10	94.1	0.05
2	(A)+200 mM NaCl	0.36	0.2	34.10	3.9	0.01
3	(A)+250 mM NaCl	10.10	5.7	12.50	1.4	0.81
4	(A)+300 mM NaCl	16.27	9.2	2.50	0.3	6.51
5	(A)+350 mM NaCl	73.53	41.5	1.44	0.2	51.06
6	(A)+400 mM NaCl	28.67	16.2	0.09	0.01	318.56
7	(A)+450 mM NaCl	7.46	4.2	0.02	<0.01	373.00
8	(A)+500 mM NaCl	2.82	1.6	0.03	<0.01	94.00
9	(A)+1000 mM NaCl	2.69	1.5	0.06	<0.01	44.83
Sum		179.74	101.5	878.84	99.9	70.23 (Mean of Fra. 5—9)

177.0 U of F VIII with 880.0 mg of total proteins were applied. a) (A): 20mM Tris-HCl (pH 7.4). b) Breakthrough.

min and ferritin. The serious effect of molecular size of absorbed proteins on the capacity of DEAE-Sphearose CL-6B is assumed to be coursed by a reduction of effective exchange area of the gel beads due to less- or non-penetration of the large size molecules into pores of the gel matrix.[6)] This result indicated a beneficial feature of DEAE-GM 10000 in the ionic exchange of large size macromolecules.

Comparison of Adsorption Capacity to Factor VIII among Various DEAE-Macroporous Spherical Gels. A representative of large molecular size proteins is blood coagulation factor VIII/von Willebrand factor complex in plasma, and DEAE-Sephadex A-50 has been applied to factor VIII preparation.[7)] By a batch-wise method with the defibrinogenated cryoprecipitate fraction, binding capacity to factor VIII was compared among five kinds of DEAE-GM, and DEAE-Toyopearl 650, EMD DEAE-650, and DEAE-Collulofine A-800. As shown in Fig. 4, DEAE-GM 10000 possessed the highest affinity and binding capacity to factor VIII. Figure 5 is a plot of correlation between Mlim of GM gels and relative adsorption capacity to factor VIII. GM gels with higher Mlim possessed higher binding capacity to factor VIII and this trend seemed to be more pronounced at Mlim larger than 2×10^6.

From these results, it was assumed that the higher adsorption capacity of the DEAE-GM gels than that of other DEAE-spherical gels was attributed to the larger pore size of the GM gel matrix. To obtain the high adsorption capacity to factor VIII in the plasma fraction, the gel matrix, at least in the case of GM gels, should bear macropores as large as at least 3×10^6 of Mlim for polyethylene glycol.

Application of DEAE-GM Column in HPLC to Factor VIII Preparation. The hard nature of GM gel is suitable for HPLC matrix. Applicability of DEAE -GM 10000 columns to HPLC preparation of factor VIII was examined. Concerning a manufacture scale preparation in future, crude (not defibrinogenated) cryoprecipitate fraction was used as the source of factor VIII. Because of the aggregating tendency of crude cryoprecipitate fraction, column-end filters with 20 μm mesh size were used. Size of the gel beads was, therefore, controlled to 32—90 μm in diameter. A representative elution chromatogram of factor VIII and its analytical data were shown in Fig. 6 and Table 2, respectively. In this case, total recovery of factor VIII as well as proteins was completely. More than 90% of the total amount of protein were present in the breakthrough fraction, whereas bulk of factor VIII stuck on the column. About one fifth of the total factor VIII did not stick on the column, we suppose, without evidences, it might be a result of binding of anionic parts of factor VIII complex due to protein–protein interactions in a dense solution. It was found that specific activities of factor VIII were higher in fractions 5—9 which were eluted with 20 mM Tris-HCl (pH 7.4) containing 350—1000 mM NaCl.

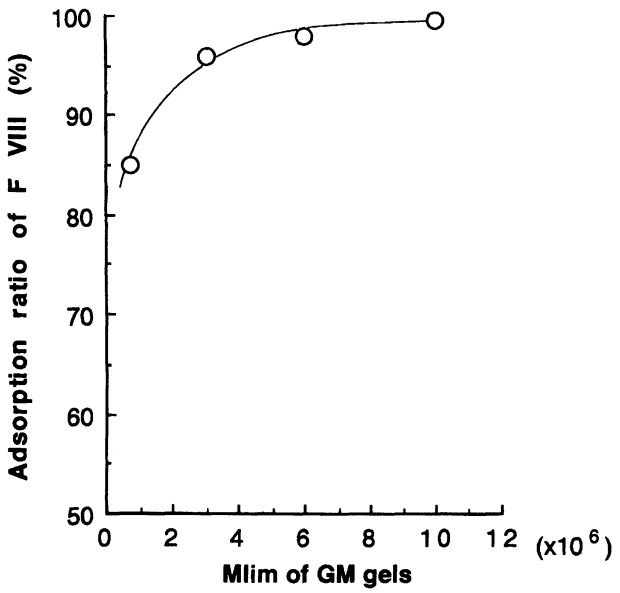

Fig. 5. Correlation between exclusion limit (Mlim) of glucomannan gels and relative adsorption capacity to factor VIII. Data of the adsorption ratio of factor VIII were determined from the results of Fig. 4.

Table 3. Column-Wise Purification of F VIII by DEAE-GM 10000

Run No.	1	2	3	4	5
Column size (mm)	6×100	6×100	6×100	6×100	6×100
Gel volume (ml)	2.83	2.83	2.83	2.83	2.83
Flow rate (L. V.)					
Adsorption ($m\,h^{-1}$)	2	1	2	2	2
Desorption ($m\,h^{-1}$)	4	4	4	4	4
Cryoprecipitate					
F VIII (U F VIII:C ml^{-1})	5.6	5.6	4.0	3.5	3.7
Proteins ($mg\,ml^{-1}$)	39.2	42.2	24.4	17.6	15.3
Specific activity (U F VIII:C/mg-P.)	0.14	0.13	0.17	0.2	0.24
Applied amount					
F VIII (U F VIII:C/ml-gel)	29.6	59.3	50.0	62.5	65.9
Proteins (mg/ml-gel)	207.8	447.3	301.8	311.0	270.3
Adsorption of F VIII					
Amount (U F VIII:C/ml-gel)	26.1	45.2	41.6	49.2	58
Ratio (%)	88.2	76.2	83.2	78.7	88.0
Recovery of F VIII					
Total recovery (%)	86.5	91.3	86.8	101.5	77.3
Recovery from column (%)[a]	50.9	61.5	61.3	82.8	52.2
Proteins					
Adsorption amount (mg/ml-gel)	24.0	24.6	21.0	18.3	18.0
Total recovery (%)	106.1	99.8	98.9	99.9	99.0
Specific activity					
Fraction 5—9 (U F VIII:C/mg-P.)	10—130	16—58	12—190	45—370	—[b]
Mean (U F VIII:C/mg-P.)	22	24	58	70	113
Purification factor (fold)	157	185	341	350	471

a) Amount of F VIII (Fra. 5—9)/Amount of F VIII adsorbed on column. b) The pool of fractions but not each fraction was measured.

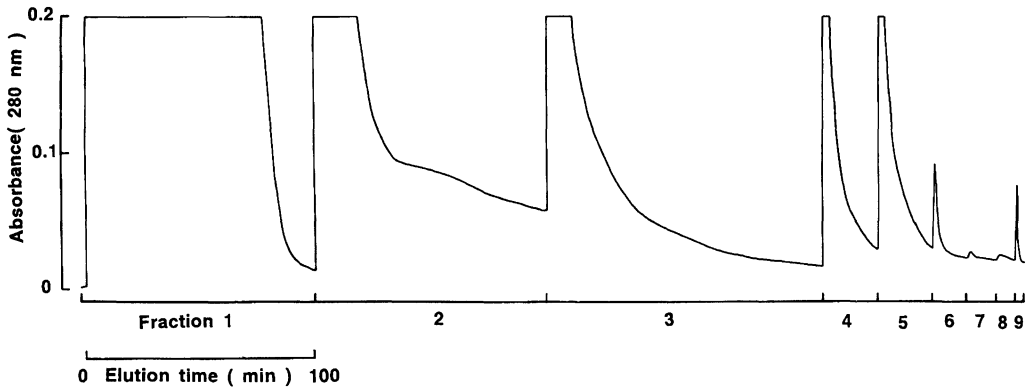

Fig. 6. Elution prophile of plasma cryoprecipitate fraction in HPLC with DEAE-GM 10000 column. HPLC conditions: Pump: LKB 2249, Column: DEAE-GM 10000 (6 mm×100 mm, bed volume 2.83 ml), Flow rate: Fraction 1 2 $m\,h^{-1}$; Fraction 2—9 4 $m\,h^{-1}$, Detector: UV, Wavelength: 280 nm.

Summary of five HPLC runs carried out under the identical conditions with repeat use of the same column was shown in Table 3. Recovery of factor VIII from the DEAE-GM 10000 column ranged from 51 to 83% (average 62%). Mean specific activities of factor VIII in the final pool fractions of the column chromatography were 22—113 U/mg-protein (average 57 U/mg-protein), and the value was largely dependent on the initial enrichness of factor VIII in the cryoprecipitate preparations. Despite such differences, the result in preparation of factor VIII with good specific activity and recovery was reproducible. Recently, affinity

chromatographies with monoclonal antibodies against factor VIII[8,9] or against von Willebrand factor[9,10] or with collagens[11] have been proposed for manufactory scale preparation of factor VIII. In these methods the macromolecules with the molecular size 150000 or more have to be immobilized on gel beads as the affinity ligands. As demonstrated presently, gel beads with very large macropores are required to express high exchange capacity in the factor VIII preparation by column chromatography even the case with small size ligand such as DEAE group. Therefore, we suspect that GM gel must be better matrix in the affinity preparations of factor VIII, too, although we have not done such a study in the present research. In our preliminary study, the tresyl-activated method was suitable to immobilize protein ligands on GM gel beads.

In any case, by the use of the monoclonal antibody column chromatography, purification of factor VIII has been achieved up to 1800 U/mg-protein as the specific activity. In comparison to such a high specific activity, the purity in the present study is just one thirtieth with the specific activity 57 U/mg-protein as the average of the 5 chromatographic runs (Table 3). However, in the affinity preparation, buffers containing albumin at the concentration of 1 mg ml^{-1} is required to maintain the activity of factor VIII. When the albumin is counted in the protein concentration according to the usual biochemical calculation, the specific activity is not 1800 U/mg-protein but 92 U/mg-protein. Therefore, in a practical sense we believe that it is worthy to consider the application of HPLC technology with DEAE-GM 10000 gel to preparation of factor VIII in manufactory scale.

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