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Synthetic sorbents for removal of factor VIII inhibitors from haemophilic A plasma

L. Dahri[a], C. Boisson-Vidal[b,*], V. Regnault[a], D. Muller[b], Y. Sultan[c], J.F. Stoltz[a]

[a]*Laboratoire d'Hémorhéologie–Hématologie, Faculté de Médecine, BP 184, 54505 Vandoeuvre lès Nancy, France*
[b]*Laboratoire de Recherches sur les Macromolécules, CNRS URA 502, Institut Galilée, Université Paris-Nord, 93430 Villetaneuse, France*
[c]*Centre de Traitement des Hémophiles, Hôpital Cochin, 75014 Paris, France*

Abstract

Human factor VIII (FVIII) is a protein of the blood coagulation system that is absent or defective in patients with haemophilia A. A most serious complication following replacement therapy in 10–15% of patients treated with available FVIII concentrate is the development of inhibitors to FVIII (anti-FVIII). Some polymers functionalized with suitable chemical substituents which mimic part of the epitope of FVIII recognized by the inhibitors might be used in extracorporeal circulation to reduce the concentration of antibodies to FVIII. For this purpose, insoluble polystyrene bearing sulfonate and L-tyrosine methyl ester sulfamide groups have been synthesized. The in vitro removal of anti-FVIII inhibitors from plasmas of patients with haemophilia A was performed. Different chromatographic parameters were studied and optimized.

1. Introduction

Hemophilia A is an X chromosome-linked disorder of blood coagulation caused by deficiency or reduction of procoagulant activity of factor VIII (FVIII). Patients with haemophilia A receive during their treatment FVIII concentrates needed to bring bleeding episodes under control. In 10–15% of polytransfused patients, antibodies to FVIII (anti-FVIII, alloantibodies) appear. Antibodies to FVIII may also arise spontaneously in post-partum women and in patients with autoimmune disorders (autoantibodies) [1]. Both types of antibodies share the ability to neutralize FVIII procoagulant activity [2,3]. With rare exceptions, anti-FVIII inhibitors have been characterized as immunoglobulins and are predominantly of IgG1 and IgG4 subclasses [4].

The management of the therapy of such patients is not well established and a variety of therapeutic options have been used [5]. Increasing the FVIII plasma level by infusion of human or porcine FVIII concentrates is the most effective method to treat an acute haemorrhage [6,7]. Plasmapheresis techniques have also been used to decrease the amount of anti-FVIII inhibitors, but this process is non-selective and other plasma proteins are also removed [8]. Intravenous administration of immunoglobulins from a large pool of plasma from normal donors to these patients has resulted in decreasing circulation of

* Corresponding author.

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autoantibodies. In this case, the anti-FVIII activity may be suppressed by anti-idiotypic antibodies present in polyspecific therapeutic IV:IgG concentrates [9].

Several affinity chromatographic methods were consequently used and developed to reduce the antibody level. In particular, columns containing protein A-coated agarose beads were used to remove these antibodies by extracorporeal circulation [10–12]. Protein A selectively binds to the Fc portions of human IgG (except for subclass 3). Nilsson and co-workers [11,12] have reported a 61–78% reduction of IgG corresponding to a decrease in the inhibitor titre of 66 to 95%. Based on the fact that anti-FVIII inhibitors are predominantly of IgG4 subclass, anti-human IgG4 antibodies were also covalently linked to agarose. Using these supports, we succeeded in eliminating large amount of anti FVIII inhibitors from circulated plasma [13].

The aim of the procedure described here was the removal of large amounts of anti-FVIII inhibitors with depletion of less normal IgG and other plasma proteins. Our purpose was to modify insoluble polystyrene resins in order to obtain specific interactions with anti-FVIII inhibitors. Previous studies have led to the synthesis of functionalized polystyrene grafted with methyl ester L-tyrosine sulfamide and sulfonate groups [14–16]. These resins, in particular those substituted by 14–30% tyrosine derivative sulfamide groups, present specific interactions with anti-FVIII inhibitors [17]. Using optimized synthesis conditions, it was possible to remove 90% of anti-FVIII from a haemophilic plasma with inhibitors [90 Bethesda units/ml (BU/ml)] in a one-step procedure under our experimental conditions [17].

We report in this paper the optimization of the in vitro removal of anti-FVIII inhibitors from the plasma of patients with haemophilia A and inhibitors to FVIII by these synthetic sorbents. Solid phases were synthesized and were tested for the effect of volume of plasma loaded, flowrates, adsorption of inhibitors and the possibility of desorption. These polymers might be used to decrease the inhibitor titre in patients using extracorporeal circulation.

2. Experimental

2.1. Sorbents

Polystyrene [Bio-Beads SX3, diameter 37–75 μm (Bio-Rad, Paris, France)] a spherical styrene–divinylbenzene copolymer with 3% cross-linking was chosen because of its good chemical and mechanical stability and the availability of functional groups for easy ligand coupling. Cross-linked polystyrene beads were chemically modified as described previously [17]. Resins differently substituted with sulfonate and methyl ester L-tyrosine sulfamide groups, PTyrOMe, were prepared and characterized (Table 1, Fig. 1).

2.2. Proteins

Plasma samples with inhibitors to FVIII were obtained from three patients. Titres of anti-FVIII inhibitors were 38, 90 and 160 BU (Centre de Traitement des Hémophiles, Hôpital Cochin, Paris, and CRTS, Nancy, France). Plasma samples were stored at −70°C before use.

2.3. Assays for anti FVIII activity

Detection of neutralizing anti-FVIII antibodies was performed using the Bethesda assay [18]. Dilutions of patients' plasma were compared with dilutions of a normal plasma pool in a one-stage kaolin-activated partial thromboplastin time (aPTT) based assay, using human factor VIII-deficient plasma as substrate. Normal human plasma was mixed with an equal volume of patients' plasma and incubated at 37°C for 2 h. Residual factor VIII levels were then mea-

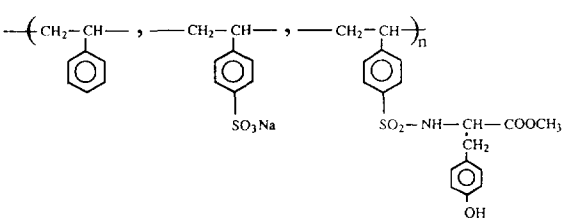

Fig. 1. Structure of functional polystyrene resins (PTyrOMe).

sured. Inhibitors were expressed in Bethesda units, which is defined as the reciprocal of the dilution of patients' plasma that inactivates half the FVIII in an equal mixture of normal and haemophilic patients' plasmas.

Detection of anti-FVIII inhibitors by an enzyme-linked immunosorbent assay (ELISA) was also performed according to a procedure described previously [19]. The ELISA method was performed to study the reactivity of haemophilia A inhibitors in comparison with their Bethesda titre.

2.4. Adsorption isotherms

The adsorption isotherms were obtained by incubating either 100 μl of purified IgG solution (0.03–5.00 mg/ml) or 50 μl of anti-FVIII inhibitors from haemophilic PPP (0.4–160.0 BU/ml) at 37°C with an equal volume of a given suspension of resin (100 mg/ml for IgG or 20 mg/ml for anti-FVIII). After incubation for 90 min, the supernatant was used to determine residual concentrations of IgG or anti-FVIII inhibitors. The assay was performed in the presence of buffer which allows the determination of initial concentrations of IgG or anti-FVIII inhibitors before adsorption.

2.5. Adsorption procedure

All the resins were suspended in phosphate-buffered saline (PBS) prior to testing. The fine particles were removed by decanting the supernatant. The remaining material (750 mg, corresponding to 1 ml of gel) was poured on to Bio-Rad columns (2 × 0.7 cm I.D.). After passivation with 1 ml of a human albumin solution at 0.5 mg/ml (CRTS), a 2-ml sample of plasma with inhibitors was loaded on to the column. The system was incubated at 37°C for various times from zero (direct elution) to 90 min, at 0.1, 1 and 2 ml/min. The fractions were collected and the residual concentration of anti-FVIII inhibitors was determined using the Bethesda method or ELISA. Columns were washed with PBS and eluted with either 10 ml of 3 *M* NaCl–0.3 *M* glycine–HCl (pH 2.8) or 2% SDS solution. Samples were immediately concentrated using a Centricon (Microconcentrators, Pharmacia, Paris, France) and studied by SDS–polyacrylamide gel electrophoresis (PAGE) using 7.5% homogeneous Phastgel (Pharmacia) following electrophoresis on a Phastsystem apparatus (Pharmacia). The standard proteins myosin (M_r 200 000), β-galactosidase (116 000), phosphorylase B (97 400), albumin (66 000) and carbonic anhydrase (45 000) were obtained from Sigma and the gels were stained for protein with silver stain solutions.

3. Results and discussion

In affinity purification, the first step is usually the immobilization of the corresponding ligand. Previous studies have demonstrated that insoluble polystyrene grafted with L-tyrosine derivatives display selective affinity towards anti-FVIII inhibitors [17]. These experiments were undertaken in order to mimic epitopes of the FVIII molecule usually recognized by the inhibitors to FVIII. It was shown that the most important constituents of our epitope were aspartic acid and tyrosyl residues [20]. Therefore, grafting of these amino acids on to polystyrene resins was decided, and the affinity of such supports for anti-FVIII inhibitors in a low-performance affinity chromatographic procedure was studied.

3.1. Adsorption isotherms in batch procedure

Anti-FVIII inhibitors and IgG adsorption isotherms were obtained on PTyrOMe15. In preliminary experiments, it was ascertained that steady-state anti-FVIII inhibitors and IgG adsorption was achieved in about 90 min at room temperature. Therefore, 90 min was chosen for the determination of the isotherms at 37°C, which are shown in Figs. 2 (anti-FVIII inhibitors) and 3 (IgG). The isotherms are of the Langmuir type in form and the data were found to provide a good fit to the Langmuir equation. On this basis, an estimate can be made of the affinity constants for anti-FVIII inhibitors or IgG binding. The application of the Langmuir model for

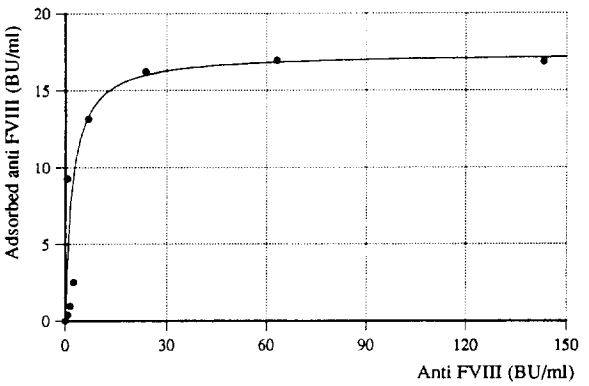

Fig. 2. Adsorption isotherm of anti-FVIII inhibitors on PTyrOMe15. A 50-μl volume of anti-FVIII inhibitors from haemophilic PPP (0.4–160.0 BU/ml) was incubated with 50 μl of resin suspension (20 mg/ml) at 37°C. After 90 min of incubation, the supernatant was used to determine residual concentration of anti FVIII inhibitors. Each point represents the average of three experiments.

the determination of equilibrium binding constants requires that the interaction be reversible in the thermodynamic sense. The plateau values are well defined and are in the range of 27 adsorbed BU/mg of resin and 0.8 mg adsorbed IgG/mg of resin. The affinity constants calculated on the basis of the Langmuir equation are $1.7 \cdot 10^8$ and $1.4 \cdot 10^5$ M^{-1} for anti-FVIII inhibitors and IgG, respectively.

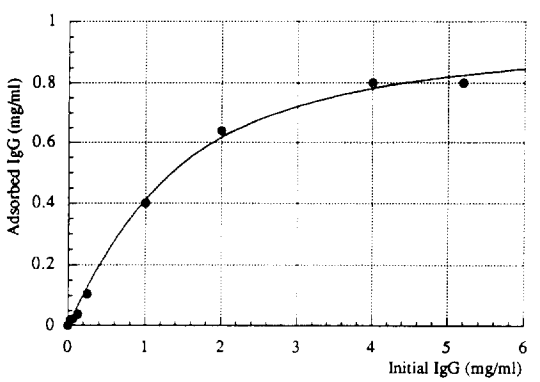

Fig. 3. Adsorption isotherm of IgG on PTyrOMe15. A 100 μl volume of purified IgG (0.03–5.00 mg/ml) was incubated with 100 μl of resin suspension (100 mg/ml) at 37°C. After 90 min of incubation, the supernatant was used to determine residual concentration of IgG. Each point represents the average of three experiments.

3.2. Adsorption in low-performance affinity chromatographic procedure

The adsorption of anti-FVIII inhibitors was studied at 37°C after a 90-min circulation time. The results obtained with all resins prepared are reported in Table 1. A good correlation between assays, the aPTT time measuring FVIII activity and the ELISA, FVIII antigen, was observed. Moreover, anti-FVIII inhibitors adsorption reached a maximum for a substitution ratio of L-tyrosine derivative sulfamide groups in the range 10–30%, corresponding to 0.8–1.3 mmol of nitrogen per gram of dry resin. The specific properties of these bioactive polymers result mainly from the random distribution of specific chemical groups on their surfaces. The conclusion of the first studies was that these bioactive polymers possessed binding sites for anti-FVIII inhibitors which ranged from specific to highly specific [21].

3.3. Chromatographic parameters involved in specific adsorption of anti-FVIII inhibitors

The flow-rate and volume of plasma loaded must be defined in order to optimize the plasma depletion yield. First, a study of the adsorption kinetics of anti-FVIII inhibitors on the functional polymers was undertaken in order to see if it is possible to reduce the incubation time at 37°C. The amount of anti-FVIII inhibitors adsorbed was calculated from the decrease in anti-FVIII inhibitors titre in the eluted fractions. Experiments were performed on different resins and showed the same results (Fig. 4). This adsorption is a slow process and the steady state is achieved in about 40 min. Considering these new results, the incubation time was reduced from 90 to 50–60 min. In clinical use, this is a gain in time of 30–40 min.

3.4. Influence of flow-rate on adsorption of anti-FVIII inhibitors

Elution flow-rates of 0.1, 1 and 2 ml/min were tested. The flow-rate of 2 ml/min corresponds to the normal flow-rate used in immunopurification

Table 1
Chemical composition of PTyrOMe resins and comparison of results obtained with adsorbed anti-FVIII inhibitors determined by the Bethesda method and ELISA

Resin	Composition (%)			PPP[a] (BU/ml)	Anti-FVIII absorbed[b] (%)	
	Φ	ΦSO_3^-	ΦSO_2 TyrOMe		Bethesda (S.D. ± 1)	ELISA (S.D. ± 3)
PTyrOMe2	26	70	4	90	83	80
				38	55	60
PTyrOMe5	15	76	9	90	95	61
				38	77	72
PTyrOMe11	20.2	67	12.8	90	94	98
PTyrOMe9	20.2	66.8	13	38	83	86
				90	nd	97
PTyrOMe10	20.2	65.2	14.6	90	88	97
PTyrOMe14	19.2	56.4	24.4	38	78	88
PTyrOMe18	19.2	56.1	24.7	38	84	91
PTyrOMe12	20.2	53.9	25.9	90	97	98
PTyrOMe16	19.2	53.9	26.9	38	80	90
PTyrOMe13	20.2	53.5	26.3	90	98	98
PTyrOMe17	19.2	48.7	32.1	38	78	91
PTyrOMe7	15	51	34	90	85	96
PTyrOMe3	15	50	35	90	80	84
PTyrOMe6	15	48	37	38	77	86
PTyrOMe8	20.2	37.6	42.2	38	91	78
PTyrOMe4	15	38	47	90	85	87
				38	63	56
PTyrOMe1	26	20	54	90	83	72
				38	65	56

[a] Inhibitor titre of haemophilic plasma tested.
[b] Amount of adsorbed anti-FVIII inhibitors determined by the Bethesda method or ELISA. The percentage of anti-FVIII inhibitors is expressed as the percentage of total amount present in the initially haemophilic plasma.

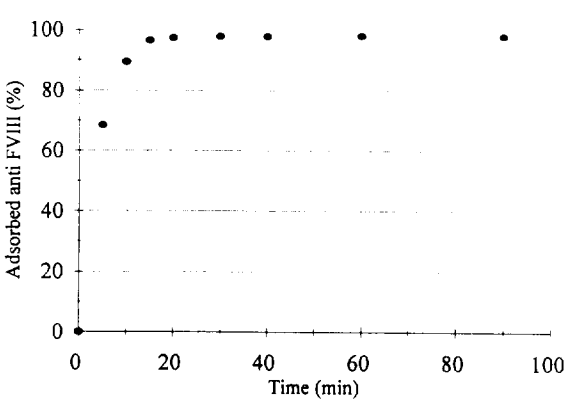

Fig. 4. Kinetics of adsorption of anti-FVIII inhibitors on PTyrOMe11 (expressed as a percentage of the total amount present in the haemophilic plasma). The inhibitor titre of loaded plasma is 38 BU/ml. Each point represents the average of three experiments.

or plasmapheresis experiments, considering the different parameters used in extracorporeal circulation: flow-rate 30 ml/h, loaded PPP/volume of gel = 2–10 and volume of gel/flow-rate = 15 min. The results are presented in Fig. 5. Adsorption of anti FVIII inhibitors is not reduced by increasing the flow-rate under our experimental conditions.

3.5. Influence of volume of plasma loaded

In order to test the adsorption capacity of the resins under dynamic conditions, several volumes of haemophilic plasma with anti-FVIII inhibitors were loaded on the column (2, 4 and 6 ml, corresponding to 76, 152 and 228 BU). The relationship between the anti-FVIII adsorption

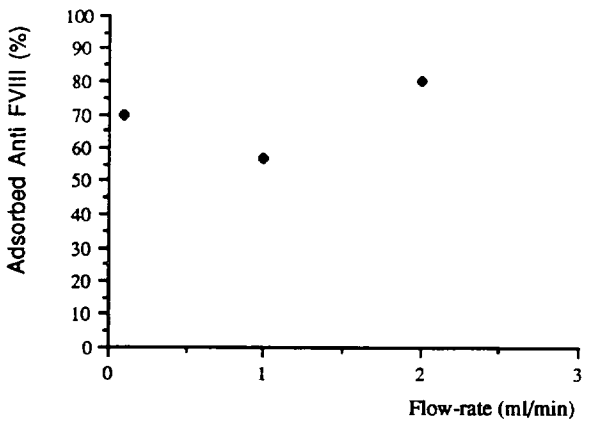

Fig. 5. Amount of anti-FVIII inhibitors adsorbed on PTyrOMe11 versus flow-rate. A 2-ml volume of haemophilic plasma was loaded on the column at a flow-rate of 0.1 ml/min, and kept under circulation for 90 min at 37°C. Each point represents the average of three experiments.

capacity and the volume of plasma loaded is linear, as shown in Fig. 6. The adsorption of anti-FVIII inhibitors on PTyrOMe resins does not reach a plateau value and the different sites present on the solid phase matrix are not saturated. On the basis of these first results, a value of 310 adsorbed BU/g is found. This value is 87 times lower than that found in the batch procedure (27 adsorbed BU/mg). This is related to the accessibility of the binding sites on the surface of the polymers, which is better in batches than in bead-loaded columns. The use of haemophilia PPP with a high level of antibodies (>500 Bu/ml) may yield more information concerning the adsorption capacity of these supports.

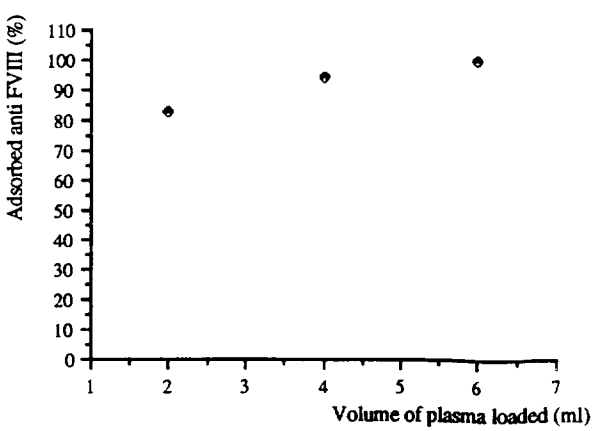

Fig. 6. Adsorption of anti-FVIII inhibitors versus volume of plasma loaded (ml). The experiment was done on PTyrOMe11 at 37°C at a flow-rate of 0.1 ml/min; 2, 4, and 6 ml of haemophilic plasma, corresponding to 76, 152 and 228 BU, were loaded on the resins. Each point represents the average of three experiments.

3.6. Regeneration of sorbents

To test if regeneration of the different resins after a plasmatic epuration is possible, each sorbent was washed with PBS. Bound anti-FVIII inhibitors were eluted using either 10 ml of 0.3 *M* glycine–HCl (pH 2.8), 3 *M* NaCl or 2% SDS solutions. The resins were then rinsed with PBS and with 1 ml of albumin solution at 0.5 mg/ml. The residual concentration of anti-FVIII inhibitors was determined after incubation of 2 ml of plasma for 90 min at 37°C. The results were compared with those obtained previously (Table 2). As can be seen, the anti-FVIII inhibitor adsorption capacity of resins treated with glycine–HCl and 3 *M* NaCl solutions is of the same order of magnitude before and after regeneration. With 2% SDS solution, a small decrease in the adsorption capacity is observed. Previous experiments showed that functionalized polystyrene resins pre-equilibrated with 4% SDS solutions adsorbed less proteins in terms of the estimated protein concentration than when they were pretreated with PBS [22]. This effect was then related to the nature of the SDS (detergent), which makes inaccessible part of binding sites from the anti-FVIII inhibitors.

The characterization of adsorbed proteins from plasma to polystyrene resins was performed on the eluted fractions. Proteins were eluted using 10 ml of 0.3 *M* glycine–HCl (pH 2.8). Fig. 7 shows the SDS-PAGE in a typical experiment using haemophilic plasma on PTyrOMe resins. The protein-staining bands were tentatively identified by their position in the gel in relation to molecular mass standards. Previous experiments on functionalized polystyrene resins showed the same gel patterns [22]. As can be seen in Fig. 7, the eluted peak shows bands at M_r 94 000 (prob-

Table 2
Anti-FVIII inhibitor adsorption capacity of three resins after regeneration by treatment with desorbing buffer and PBS

Resin	Desorbing buffer	Anti-FVIII adsorbed[a] (%)	
		A	B
PTyrOMe14 (24.4%)	0.3 *M* glycine–HCl	78	78
PTyrOMe16 (26.9%)	3 *M* NaCl	80	78
PTyrOMe17 (32.1%)	2% SDS	78	63

The titre of the initially loaded plasma tested was 38 BU/ml.
[a] Amount of adsorbed anti-FVIII determined by the Bethesda method, (A) after elution of 2 ml of haemophilic plasma and (B) after regeneration of the resin and reinjection of 2 ml of the same plasma. The percentage of anti-FVIII inhibitors is expressed as the percentage of total amount present in the initially haemophilic plasma.

ably plasminogen), 80 000 (transferrin) and 69 000 (albumin). In addition, a group of bands centred at about M_r 200 000 may be attributed to fibronectin. The main components, however, are fibrinogen (some of which is degraded) and albumin. There are major proteins of blood and are known to have a high surface affinity [23]. The adsorption of other plasma proteins is fairly low. However, despite these results, the determination of the residual concentration of eluted proteins showed a small decrease in the total amount in plasma (not more than 20%) [24].

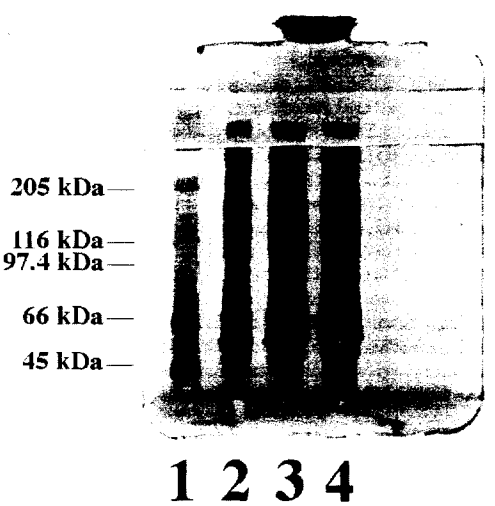

Fig. 7. SDS-PAGE (7.5% homogeneous, reducing conditions) showing comparison of 0.3 *M* glycine–HCl eluates after incubation with 2 ml of plasma (flow-rate 0.1 ml/min at 37°C) on PTyrOMe8, -9, and -10. Lanes: 1 = high-molecular mass-standards; 2 = PTyrOme8; 3 = PTyrOMe9; 4 = PTyrOMe10.

4. Conclusion

A new type of synthetic sorbent was synthesized and showed highly specific and reversible interactions with anti-FVIII inhibitors. The experiments reported were carried out in order to optimize the epuration conditions of haemophilic plasma with inhibitors. Previous results have shown that the anti-FVIII inhibitors bind to resins through their antigen binding site [16]. Considering the parameters studied, the resins are suitable for plasmatic epuration. These resins exhibit a high affinity for anti-FVIII inhibitors (10^8 M^{-1}), and a small depletion of normal IgG (<10%) or other plasmatic proteins (<20%) was observed. Even under the conditions of an extracorporeal circulation, the selectivity is still high (2 ml/min under our experimental conditions, 6 ml of plasma loaded). Moreover, these resins can be regenerated using 0.3 *M* glycine–HCl or 3 *M* NaCl solution, and their affinity for antibodies to FVIII remains similar. No degradation of the resin was observed even after numerous epurations. With suitable resins the projected application is to develop a method to remove antibodies before FVIII concentrate infusion in patients with FVIII

inhibitors. An ex vivo experiment remains to be done.

References

[1] F.M. Gill, *Prog. Clin. Biol. Res.*, 150 (1984) 19.
[2] D. Green and K. Lechner, *Thromb. Haemost.*, 45 (1981) 200.
[3] C.K. Kasper, *Prog. Hemost. Thromb.*, 9 (1987) 57.
[4] C.A. Fulcher, S.D.G. Mahonney and T.S. Zimmerman, *Blood*, 69 (1987) 1475.
[5] B.G. Mack, *Semin. Thromb. Hemost.*, 19 (1993) 13.
[6] M. Mannucci, *Thromb. Haemost.*, 70 (1993) 17.
[7] R.D. Bona, M. Riberio, A.U. Klatsky, S. Panek, M. Magnifico and F.R. Rickles, *Semin. Hematol.*, 30 (1993) 32.
[8] C. Negrier, M. Dechavanne, F. Afonsi and P.J. Tremisi, *Acta Haematol.*, 85 (1991) 107.
[9] Y. Sultan, F. Rossi and M.D. Kazatchkine, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 828.
[10] H. Lee, D. Tucker and J.P. Allain, *Thromb. Res.*, 14 (1979) 925.
[11] I.M. Nilsson, S.B. Sundqvist and C. Freiburghaus, *Prog. Clin. Biol. Res.*, 150 (1984) 225.
[12] I.M. Nilsson, E. Berntorp and C. Freiburghaus, *Thromb. Haemost.*, 70 (1993) 56.
[13] V. Regnault, C. Rivat, J.P. Vallet, C. François-Gérard, G. Brochier and J.P. Allain, *Thromb. Res.*, 45 (1987) 51.
[14] N. Bellatar, D. Gulino, J. Jozéfonvicz and Y. Sultan, in E. Chiellini, P. Guisti, C. Migliaresi and L. Nicolais (Editors), *Proceedings of the 2nd International Conference on Polymers in Medicine*, Plenum Press, New York, 1986, p. 127.
[15] H. Messaïkeh, N. Bellatar, D. Gulino, Y. Sultan and J. Jozéfonvicz, in D. Christel, A. Meunier and A.J.C. Lee (Editors), *Biological and Biomechanical Performance of Biomaterials*, Elsevier, Amsterdam, 1986, p. 321.
[16] C. Boisson-Vidal, H. Messaïkeh and J. Jozéfonvicz, *J. Mater. Sci.: Mater. Med.*, 2 (1991) 193.
[17] L. Dahri, C. Boisson-Vidal, D. Muller and J. Jozéfonvicz, *J. Biomater. Sci., Polym. Ed.*, (in press).
[18] C. Kasper, L.M. Aledort, R.B. Counts, J.R. Edson, J. Fratantoni, D. Green, J.W. Hampton, M.W. Hilgartner, J. Lazerson, P.H. Levine, C.W. McMillan, J.G. Pool, S.S. Chapiron, N.R. Shulman and J. Van Eys, *Thromb. Diath. Haemorrh.*, 34 (1975) 875.
[19] V. Regnault and J.F. Stoltz, *Blood*, 83 (1994) 1155.
[20] M. Shima, C.A. Fulcher, S.D.G. Mahonney, R.A. Hougten and T.S. Zimmerman, *J. Biol. Chem.*, 261 (1988) 10198.
[21] J. Jozéfonvicz and M. Jozéfowicz, *Pure Appl. Chem.*, 64 (1992) 1783.
[22] C. Boisson-Vidal, J. Jozéfonvicz and J.L. Brash, *J. Biomed. Mater. Res.*, 25 (1991) 67.
[23] L. Vroman, A.L. Adams, G.C. Fisher and P.C. Munoz, *Blood*, 65 (1980) 156.
[24] M. Haouam and Y. Sultan, personal communication.