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Application of a new statistical approach to optimize the immunopurification of antihemophilia A factor

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ABSTRACT

Our aim was to optimize the immunopurification process of human factor VIII. This purification was performed using a mouse monoclonal anti-factor VIII light-chain antibody. Previous dissociation of the factor VIII–von Willebrand factor complex with $CaCl_2$ led to a 50% increase of the factor VIII adsorption on the immunosorbent. The optimization of the elution step required the analysis of the effects of two parameters, pH and ionic strength, on four different responses: elution yield, concentration, specific activity and stability of factor VIII. For this purpose, a multifunctional method using Doehlert matrices for statistically designed experiments was applied. This methodology allowed us to obtain, with only seven experiments, a 60% increase of the elution yield and a two-fold increase of the specific activity of factor VIII.

INTRODUCTION

Antihemophilia A factor (FVIII) is a glycoprotein that acts in the coagulation pathway as a cofactor of factor IXa to activate factor X. Its deficiency or absence causes a serious bleeding disorder, hemophilia A. FVIII circulates in plasma at very low concentration (100 ng/ml) and is complexed to another protein, the von Willebrand factor (vWf). Furthermore, FVIII comprises different active heterodimers in the M_r ranges 80 000–210 000 and 80 000–90 000 [1]. Because of all these characteristics, a very specific purification process is needed to obtain the different forms of FVIII. Among the various procedures described for the isolation of FVIII [2], classical purification processes always give low yields because of the numerous steps involved. By contrast, a one-step immunopurification process using monoclonal antibodies against vWf or FVIII has been successfully performed to produce highly purified FVIII with higher yields

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[1,3–10]. These antibodies bind to the FVIII–vWf complex by the recognition of an epitope on either vWf [4–9] or FVIII [3,6,9–10]. Nevertheless, immunopurification using anti-vWf antibodies needs large amount of immunosorbent, owing to the presence of free vWf in the commercial concentrates (the ratio FVIII/vWf was 1:6). Because of the higher capacity of an anti-FVIII immunosorbent to bind FVIII, the immunopurification process described in this paper has been performed with a mouse monoclonal anti-factor VIII antibody (CAG1-463-A8) [11].

The aim of this work was to optimize the two steps of the immunopurification process: the adsorption and the elution of FVIII from the support. During the adsorption phase, the recognition of the antibody to the corresponding epitope on the FVIII light chain should be altered by the presence of vWf. Indeed, Hamer *et al.* [12] and Lollar *et al.* [13] have shown that FVIII is complexed to vWf via its light chain. Thus, we have studied the binding of the protein to the immobilized CAG1-463-A8 antibody after dissociation of the FVIII–vWf complex with $CaCl_2$ [14].

The optimization of FVIII elution from the immunosorbent requires a detailed analysis of multiple responses (total amount, concentration, specific activity and stability of immunopurified FVIII) obtained by the variation of different parameters (such as pH and ionic strength). This study requires a large number of experiments, a long time, and considerable difficulty in the interpretation of the results. In order to improve the quality of the investigation and to save time, we present in this paper a multifactorial analysis of the purification conditions using Doehlert matrices for design experiments [15]. This statistical method, widely described for the definition of culture media [16,17], represents a new approach in chromatography.

EXPERIMENTAL

Samples

FVIII was immunopurified from concentrates produced by the Centre National de Transfusion Sanguine (CNTS, Les Ulis, France). The monoclonal antibody AMC-463 (CAG1-463-A8) elicited against FVIII light chain [11,18] was purified from mouse ascites using a DEAE Affiblue gel column (Bio-Rad, Richmond, CA, USA).

Factor VIII assays

The FVIII procoagulant activity was measured by the "one-stage clotting" assay using a kaolin-activated method [19] with FVIII concentrate from CNTS as standard.

The von Willebrand factor antigen was determined by enzyme-linked immunosorbent assay (ELISA) using a sandwich technique (Asserachrom vWF, Diagnostica Stago, Franconville, France).

Protein concentrations were determined according to Bradford [20] using bovine serum albumin (BSA, Bio-Rad) as a standard.

The specific activity (SA) was calculated as the ratio of the coagulant activity ("one-stage clotting" assay) to 1 mg of protein.

Sodium dodecyl sulphate polyacrylamide gel elecrophoresis (SDS-PAGE) was performed as described by Laemmli [21]. A 3% (w/v) stacking gel with a 6–12% (w/v) gradient separating gel was used. After the addition of 10% (w/v) SDS (30 μl) and bromophenol blue (10 μl), the samples (190 μl) were heated for 5 min at 95°C under reducing conditions and applied to the gel. A mixture of high- and low-molecular-mass proteins (Bio-Rad) was used as markers. Gels were run at 70 V for 16 h and silver-stained [22]. Gels were then read with a laser scanning densitometer (Preference, Sebia, Issy les Moulineaux, France).

Immunopurification of FVIII

FVIII was purified using a mouse monoclonal anti-factor VIII antibody (CAG1-463-A8).

Immunosorbent matrix. The monoclonal antibody was immobilized on a CNBr-activated Sepharose 4B gel (Pharmacia) at a density of 0.5 mg immunoglobulin G (IgG) per ml of gel, as previously described [11]; 500 U of FVIII were added to 1 ml of immunosorbent. FVIII concentrates were dissolved at 37°C in buffer A (0.02 *M* imidazole, 0.15 *M* NaCl, 0.01 *M* $CaCl_2$, 0.1 *M* lysine, 2.3 mM diisopropyl fluorophosphate) at pH 6.8 and then applied to the support. Before adsorption, the gel was equilibrated with buffer A.

Optimization of immunoadsorption. In order to optimize the FVIII fixation to immunosorbent, 0.25 M $CaCl_2$ was added to buffer A to dissociate FVIII from vWf [14,23]. The efficiency of the dissociation was ascertained by gel permeation on Sepharose CL4B (XK26/100, Pharmacia).

Kinetic of FVIII adsorption on the immunosorbent

Batchwise adsorption of FVIII on the immunosorbent in dissociative conditions for the FVIII–vWf complex was achieved at room temperature during 24 h. Aliquots of the suspension (100 μl) were centrifuged every hour (1000 g, 1 min), and the coagulant activity was measured in the supernatant to determine the extent of adsorption. vWf antigen and protein concentrations were also measured in each aliquot. Kinetic of FVIII adsorption in non-dissociative conditions of FVIII–vWf complex was also achieved as a reference.

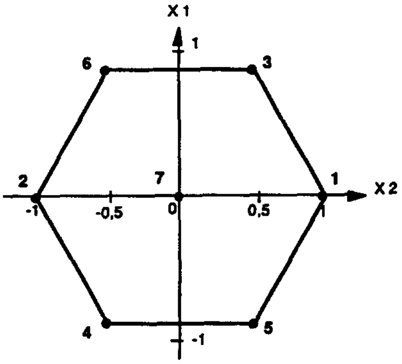
Optimization of FVIII elution

Previous studies have tested a range of possible eluents of FVIII from monoclonal antibodies [4,24,25]. In this study buffer A contained 50% ethylene glycol, 10 mM benzamidine and 2 mM phenylmethylsulphonyl fluoride, (pH 6.0). Optimization of these elution conditions required a detailed analysis of the effects of pH and ionic strength. For this study, we used a statistical method [15].

Mathematical model. The Doehlert uniform shell design of experiments was used. This method is derived from the simplex method, and represents a spacial optimization technique over a defined experimental domain (rhombic shell). The number of experiments (n) to perform is related to the number of variables (K) to test by the following equation: $n = K^2 + K + 1$ (for $K = 2$, $n = 7$). The Doehlert matrix of a design for two variables is shown in Table I.

TABLE I

DOEHLERT UNIFORM SHELL DESIGN FOR TWO VARIABLES ($K = 2$)



Experiment No.	Theoretical values	
	X_2	X_1
1	1	0
2	−1	0
3	0.5	0.866
4	−0.5	−0.866
5	0.5	−0.866
6	−0.5	0.866
7	0	0

A theoretical model was used for the mathematical analysis of the experimental responses (Y). Considering that there is no discontinuity and that the surface response has only one maximum in the defined domain, a second-order polynomial model was chosen [25]. For two parameters (X_1 and X_2), this model can be written:

$$Y_i = B_0 + B_1X_{1i} + B_2X_{2i} + B_{11}X_{1i}^2 + B_{22}X_{2i}^2 + B_{12}X_{1i}X_{2i} \quad (1)$$

The six B_i coefficients that represent the evolution of the response over the domain are calculated from the seven experimental responses (Y_i). For each B_i coefficient, a mean value was determined. Using these coefficients and eqn. 1, calculated responses (Y_{cal}) were obtained and compared with experimental values (Y_{exp}). If the difference between the experimental and theoretical values was within the range of error of the method used for Y quantification, the model was accepted (for biological systems, a range of 10% is acceptable). In this case, it is possible to predict the responses in each point of the domain. A the-

TABLE II

VARIABLES, pH AND IONIC STRENGTH, AND CORRESPONDING VALUES OBTAINED FROM THE DOEHLERT MATRIX

Experiment No.	Experimental values	
	pH	NaCl concentration
1	8.0	1.0 *M*
2	6.0	1.0 *M*
3	7.5	1.87 *M*
4	6.5	0.13 *M*
5	7.5	0.13 *M*
6	6.5	1.87 *M*
7	7.0	1.0 *M*

oretical optimum can be defined and has to be validated by an experimental measurement in the corresponding conditions.

Application. To optimize the elution conditions, the pH and ionic strength have been identified. The pH (X_2) varied between 6 and 8 while the ionic strength (X_1) ranged from 0 to 2 *M* NaCl, which is consistent with FVIII stability. The Doehlert experimental matrix is shown in Table II.

After FVIII had bound to the immunosorbent, the gel was washed with buffer A and separated into seven equal volumes. In each condition, FVIII was eluted at 22°C and at a flow-rate of 400 μl/min. Four different responses (Y) were measured:

(1) the total amount of FVIII eluted from the column;

(2) the concentration of FVIII;

(3) the specific activity of the immunopurified product;

(4) the stability of FVIII during 24 h at 4°C.

The elution buffer was then removed by gel permeation using a G25 Sephadex column (Pharmacia) in a 20 m*M* Tris buffer (pH 7.2) containing 0.15 *M* NaCl and 10 m*M* $CaCl_2$ (buffer B).

Column liquid chromatography characterization

Immunopurified FVIII was subjected to fast protein liquid chromatography (FPLC) on an anion-exchanger Mono Q column (HR 5/5, Pharmacia) equilibrated with buffer B and eluted with a gradient (0.15 to 1 *M* NaCl) at a flow-rate of 0.5 ml/min at room temperature. Eluted fractions were analysed by SDS-PAGE and tested for coagulant activity [26].

RESULTS AND DISCUSSION

Immunoadsorption of FVIII

We have studied the immunoadsorption of FVIII in both non-dissociative and dissociative conditions of the FVIII–vWf complex. Fig. 1a shows the biphasic kinetics of FVIII adsorption on the immunosorbent in non-dissociative conditions (10 m*M* $CaCl_2$). After 4 h of incubation, 54% of FVIII was bound to the support and no significant increase of FVIII binding was noted during the next 2 h. Similar biphasic kinetics were obtained in the presence of 250 m*M* $CaCl_2$, which dissociates the FVIII–vWf complex. Nevertheless, we observed a significant increase of the yield of immunoadsorption (Fig. 1b): the maximum reached 83% after 4 h.

Fig. 1 also shows the kinetics of vWf adsorp-

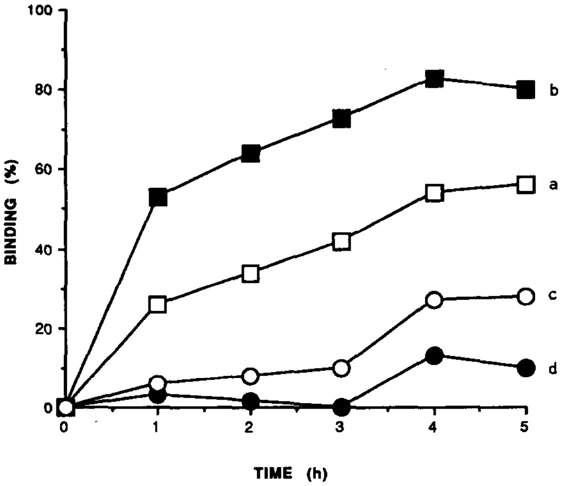


Fig. 1. Kinetic adsorption of FVIII and vWf on anti-FVIII light-chain immunosorbent, under non-dissociative conditions (10 m*M* $CaCl_2$) and under dissociative conditions (250 m*M* $CaCl_2$) of the FVIII–vWf complex. (a) FVIII adsorption in presence of 10 m*M* $CaCl_2$ (□). (b) FVIII adsorption in presence of 250 m*M* $CaCl_2$ (■). (c) vWf adsorption in presence of 10 m*M* $CaCl_2$ (○). (d) vWf adsorption in presence of 250 m*M* $CaCl_2$ (●).

TABLE III

OPTIMIZATION OF HUMAN FVIII ELUTION USING A DOEHLERT MATRIX: EXPERIMENTAL RESPONSES

Experiment No.	Volume (ml)	Activity (I.U./ml)	Amount (I.U.)	Proteins (mg/ml)	Specific activity (I.U./mg)	Stability over 24 h at 4°C
1	7	229	1603	0.056	4089	0.74
2	6	216	1296	0.062	3484	0.73
3	5	342	1710	0.062	5516	0.60
4	5	265	1325	0.056	4732	0.99
5	8	141	1128	0.048	2938	0.99
6	6.5	259	1684	0.056	4625	0.89
7	7	206	1442	0.074	2784	0.86

tion in non-dissociative (Fig. 1c) and dissociative (Fig. 1d) conditions. In the presence of 10 m*M* $CaCl_2$, we observed continuous binding of vWf with a maximum of 27%. By contrast, in presence of 250 m*M* $CaCl_2$, no significant fixation of vWf was noted before 3 h and only 10% after 6 h. This result indicates that the dissociation of FVIII–vWf complex decreased the binding of vWf to the gel, suggesting that vWf was fixed to the immunosorbent via FVIII.

Our data revealed that FVIII immunoadsorption depends on the association of FVIII with vWf. If this FVIII-vWf complex was not previously dissociated, vWf decreased the fixation of FVIII to the anti-light-chain antibody (64%). Since vWf binds to the light chain of FVIII [27–29], the low yield of FVIII adsorption observed in the presence of vWf can be explained by steric hindrance due to this protein, which diminishes the interaction between FVIII and the immobilized anti-light-chain antibody.

Factor VIII elution

Different pH and ionic strength conditions were screened for the optimization of FVIII elution. This was performed by choosing Doehlert matrices and a second-order polynomial model to represent the four responses (yield, concentration, specific activity and stability). Computer analysis of the experimental data, summarized in Table III, allowed us to determine the validity of the model and to define the B_i coefficients for the calculation of the different theoretical responses in the entire domain. The isoresponse curves are shown in Fig. 2.

Optimization of the yield. Since the difference between the experimental and theoretical responses was only 4%, the polynomial model reflected the experimental phenomenon. The isoresponse curve (Fig. 2a) showed that, at each pH value, the elution yield increased with osmolarity. Since the variation of coagulant activity between two isocurves was 70 I.U., Fig. 2a showed that at pH 6, this variation corresponded to only 280 I.U. (between 0 and 2 *M* NaCl), whereas it reached 700 I.U. at pH 8 (between 0.15 and 1.6 *M* NaCl), indicating that at high pH the response is more sensitive to the ionic strength effect. At lower NaCl concentration (0–1 *M* NaCl), Fig. 2a did not show a significant variation of the elution yield at all pH values of the domain. This is characterized by isocurves parallel to the *y*-axis. By contrast, for 1–2 *M* NaCl, we observed a synergic effect of the two parameters (pH and ionic strength) on the response. The lowest yield (48%) was obtained at pH values ranging from 7 to 8 and NaCl concentrations below 0.15 *M*. In the experimental domain defined by dashed lines in Fig. 2a (pH 7–8, 1.7–2 *M* NaCl), the theoretical elution yield reached a maximum of 75%. However, SDS-PAGE revealed total FVIII elution at 2 *M* NaCl (data not shown).

Optimization of FVIII concentration. The same study was performed to optimize the FVIII con-

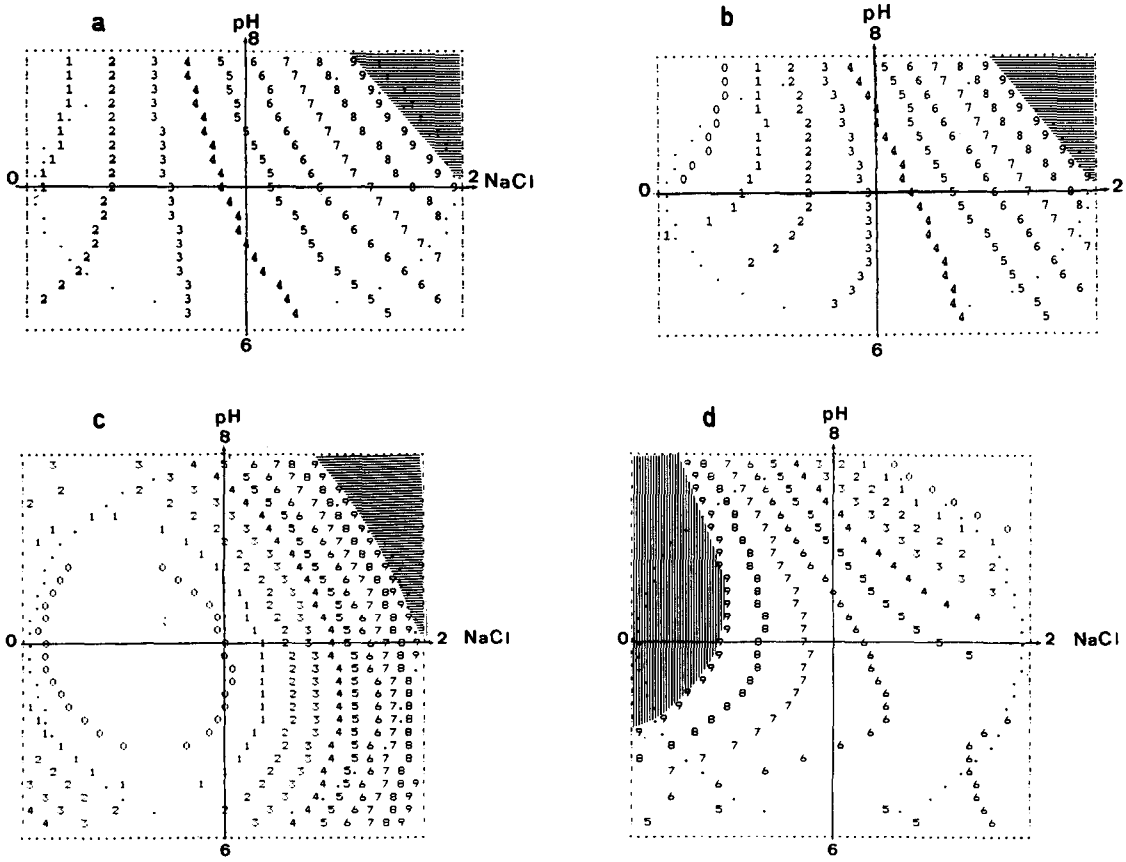


Fig. 2. Effects of pH and ionic strength on elution of FVIII. Over the domain, variations of the responses were defined by different curves of isoresponses increasing from 0 to 9. The dashed lines show the domain of maximum response. (a) Elution yield isocurves. (b) Concentration isocurves. (c) Specific activity isocurves. (d) Stability isocurves.

centration. A second-order polynomial model can be also used to represent the experimental phenomenon (difference of 4% between experimental and theoretical responses). The isoresponse curves corresponding to the effects of pH and ionic strength (Fig. 2b) were very similar to the preceding ones (Fig. 2a). Indeed, the FVIII concentration increased with the osmolarity at each pH value, and a synergic effect was also identified for NaCl concentration from 1 to 2 *M*. As observed for the elution yield, the FVIII concentration was more sensitive to the osmolarity than to the pH of the buffer. Considering that the variation of the coagulant activity between two isocurves was 20 I.U./ml, the variation of the response was 80 I.U./ml at pH 6 (0–2 *M* NaCl; Fig. 2b), whereas at pH 8 it reached 180 I.U./ml (0.3–1.5 *M* NaCl; Fig. 2b). The maximum FVIII concentration (C_{max}) is represented by the dashed lines in Fig. 2b (pH 7–8, 1.6–2 *M* NaCl).

Optimization of the specific activity. Considering that the differences between calculated and experimental specific activities were less than 2%, the mathematical model was validated. Contrary to the previous responses, Fig. 2c showed that over a large domain (0–1 *M* NaCl and pH 6–8), the theoretical model predicted a weak specific activity. We also observed (Fig. 2c), for pH values lower than 7 and ionic strength values ranging from 1 to 2 *M* NaCl, that the increase of the specific activity depended only on the variation of the buffer osmolarity. By contrast, at high pH

(7–8), we noted a synergic effect of the two parameters on the repsonse. The specific activity reached the maximum of 5500 I.U./mg (dashed lines, Fig. 2c). Considering that the variation of the specific activity between two isocurves was 275 I.U./mg, a two-fold increase of this response was achieved over the domain.

Optimization of the stability of FVIII. The comparison of theoretical and experimental responses (8%) allowed us to validate the second-order polynomial model chosen to describe the stability of eluted FVIII. Contrary to the previous responses, the stability of FVIII increased with decreasing NaCl concentration (Fig. 2d). We also noted that this stability was more sensitive to the osmolarity variation at pH 8 than at lower pH. For pH values ranging from 7 to 8 and concentrations above 1 *M* NaCl, we observed a synergic effect of these two parameters to diminish FVIII stability. By contrast, for NaCl concentrations between 0 and 1 *M*, no significant influence of the pH on the response was observed (Fig. 2d). The maximum of the response was observed at pH 6.5–8 and NaCl concentrations of 0–0.5 *M* (Fig. 2d, dashed lines). Since the difference between two isocurves corresponded to a variation of the stability of 4% (Fig. 2d), the variation over the total domain was only 40%.

Validation of the optimal response. The optimal elution conditions defined for FVIII yield, concentration and specific activity led to a high instability of the product. Thus the compromise for optimal theoretical response was obtained by the overlay of the different isocurves (Fig. 2). The elution buffer containing 2 *M* NaCl at pH 7.0 was chosen to maintain the maximum response for yield, concentration and purity, and to limit the instability of purified FVIII. These theoretical conditions have been tested. The elution yield was 70%, and the product was concentrated 17 times and purified 4000 times (SA = 5000 I.U./mg) compared with the starting material. Furthermore, the FVIII activity recovered after 24 h at 4°C was 80%. These results are consistent with the theoretical response.

FPLC characterization

FVIII immunopurified in the conditions optimized by the experimental matrix of Doehlert was subjected to FPLC and SDS-PAGE analysis. As previously described [26], this method allowed us to separate the different heterodimers of FVIII in the M_r ranges 80 000–210 000 and 80 000–90 000. This step led to a three-fold concentration of the product and an increase of its specific activity to 9000 U/mg. This characterization suggested that the FVIII immunopurified under these new conditions was not modified compared with the FVIII previously described [26].

CONCLUSION

Optimization of the pH and ionic strength, which are the parameters that have most effect on the elution of FVIII, has been performed using Doehlert matrices to improve the elution yield, concentration, specific activity and stability of FVIII. This methodology saves time and reagents, and greatly improves the efficiency and the quality of the investigation with only seven experimental data. Despite the reduced number of experiments, it was possible to obtain an infinity of theoretical responses over all the defined domain. Furthermore, this methodology revealed the synergic effects of the two parameters on the response.

The interpretation of the different isocurves allowed us to define a new elution buffer, leading to a 60% increase of the elution yield and a two-fold increase of the specific activity. Such an approach has previously been used for the optimization of culture media [30]. We have proved the efficiency of the method for purification purposes. This method should be used more frequently in the optimization of processes that are multiparametric.

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