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Optimization by factorial analysis of caprylic acid precipitation of non-immunoglobulins from hyperimmune equine plasma for antivenom preparation

B.C. Nudel [b], C. Perdoménico [a], R. Iácono [a,b], O. Cascone [a,b,*]

[a] ANLIS Malbrán, Instituto Nacional de Producción de Biológicos INPB, Vélez Sársfield 563, (1281) Buenos Aires, Argentina
[b] Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, (1113) Buenos Aires, Argentina

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ABSTRACT

Optimization of caprylic acid precipitation of equine plasma non-immunoglobulin proteins for antivenom preparation was achieved by regression analysis of the responses of three highly significant factors assayed by factorial design. The factors studied were caprylic acid concentration, plasma pH and temperature, and their response was assessed in terms of filtration speed, residual albumin, total protein content and turbidity. The results evidenced that the three variables are involved in the precipitation process. Moreover, the factors displayed significant interactions, indicating that their levels distinctly affect the optimization procedure. The best combination was 3% caprylic acid, 37 °C and plasma pH 4.9; under these conditions, all immunoglobulins and only 0.1% albumin remained in the supernatant, in a very fast and simple procedure. After formulation, the antivenom obtained by this procedure presented full lethality neutralizing activity and absence of protein aggregates.

1. Introduction

Formerly, antitoxic sera were widely used in the treatments of diphtheria, tetanus and snakebite envenomation. Although vaccination has replaced the use of hyperimmune sera for diphtheria and tetanus, the main resource against snakebite envenomations is still the antivenom obtained from hyperimmune horse plasma, which consists of whole IgG or its $(Fab')_2$ fragment (Morais and Massaldi, 2009).

The use of whole IgG antivenom has been questioned, arguing that its Fc fragment is mainly responsible for the early adverse reactions associated with the i.v. administration of these sera (Chippaux, 1998). Taking this assumption into account, in many countries, antivenoms are prepared by IgG digestion with pepsin to obtain the $(Fab')_2$ fragment. However, Arroyo et al. (1999); Otero-Patiño et al. (1998) and Otero et al. (1999) reported that the administration of whole IgG antivenoms produce a relatively low incidence of early adverse reactions. Instead, the presence of protein aggregates, the contaminant proteins and the total load of heterologous proteins may be the main cause of the unwanted adverse reactions (León et al., 2001; Lalloo and Theakston, 2003). Thus, the antivenom production process should be specific and, taking into account that snakebites occur mainly in poor countries, it should also be cost-effective.

Several methods have been developed for antivenom production from hyperimmune animal plasma. Chromatographic techniques, such as affinity and ion exchange, yield a product of high quality (Sullivan and Russell, 1982; Russell et al., 1985; Dias et al., 1989; Smith et al., 1992), but the cost is rather high and

* Corresponding author. Campichuelo 103, (1405) Buenos Aires, Argentina. Tel.: +54 11 4964 8269; fax: +54 11 4508 3645.
E-mail addresses: osvaldocascone@yahoo.com.ar, ocasco@ffyb.uba.ar (O. Cascone).

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requires specialized equipment. Methods based on IgG or its $F(ab)'_2$moiety precipitation with ammonium sulfate usually bring about yield loss and the possibility of aggregates formation.

Non-immunoglobulin protein precipitation by caprylic acid has been used for a long time (Steinbuch and Audran, 1969; McKinney and Parkinson, 1987; Perosa et al., 1990). In 1994, Rojas et al. reported an excellent method for antivenom production from hyperimmune horse plasma by an optimized single precipitation step with caprylic acid that gives a highly enriched IgG preparation with high yield and good neutralizing activity. As IgG always remains soluble, the yield loss and the possibility of aggregates formation of this method are lower. The variables studied by these authors were the caprylic acid concentration, the pH and the stirring rate and intensity.

At low pH, the hydrophobicity of the octyl moiety of caprylic acid dominates and makes acidic proteins such as albumin in the solution precipitate. Antibodies with basic pI have sufficient charge to counteract that hydrophobicity and remain in the supernatant (Wang et al., 2009).

Since hydrophobic interaction is mandatory in caprylic acid precipitation of albumin, and it is well known that temperature has an important influence on this type of interaction (Baldwin, 1986; Haidacher et al., 1996), we included this parameter in addition to caprylic acid concentration and pH in a factorial design in order to optimize the precipitation. This design also allowed assessing the interaction between these variables. Surprisingly, some interactions not previously envisioned strongly influenced the purification performance.

2. Materials and methods

2.1. Venom and crotalic hyperimmune plasma

Venom was obtained from snakes collected in Argentina, and then lyophilized and kept at −20 °C until use. After immunization of horses with venom of *Crotalus durissus terrificus* for 60 days following the standard schedule, blood was withdrawn on sodium citrate and the plasma separated after decantation at 4 °C.

2.2. Caprylic acid precipitation

The variables studied were: caprylic acid concentration, pH and temperature.

Aliquots of 50 ml of hyperimmune horse plasma were brought to the desired pH with 1 N HCl. After the temperature was brought to the allotted value, caprylic acid was added to reach the desired concentration, under vigorous stirring for 1 h. It is well known that vigorous stirring is required for an effective precipitation of non-IgG proteins (Rojas et al., 1994).

2.3. Filtration assay

After precipitation, the suspension was brought to room temperature (RT) and filtered through Whatman 1 paper. The time to obtain 10 ml filtrate was arbitrarily recorded as filtration speed.

2.4. Total protein and albumin concentration measurements

Total protein concentration in the final formulation was measured by the Biuret technique (Gornall et al., 1949) and that in the precipitation supernatants by the Bradford method (Bradford, 1976). Caprylic acid in the supernatant interferes with the protein measurement by Biuret reagent. Albumin concentration was measured by the bromocresol green method (Doumas et al., 1971) with the Albúmina reagent from Wiener Labs, Argentina.

2.5. Caprylic acid concentration measurement

Caprylic acid concentration was measured by the procedure developed by Lowry and Tinsley (1976) for the measurement of organic acids.

2.6. Protein aggregates

Samples of 2 μl antivenom were loaded to a BioSep S-4000 HPLC column (7.8 × 300 mm; Phenomenex Inc., Torrance, CA, U.S.A.) coupled to a Shimadzu Prominence HPLC equipment (Shimadzu Corp., Japan). The column was equilibrated and eluted with 100 mM sodium phosphate buffer, 0.85% NaCl, pH 7.0. The flow rate was 1 ml/min and proteins were detected by their absorbance at 280 nm.

2.7. Turbidity measurement

Turbidity was measured by the absorbance at 600 nm (Rojas et al., 1993) in an Ultrospec 2000 spectrophotometer (Pharmacia Biotech, Uppsala, Sweden).

2.8. SDS-PAGE

SDS-PAGE was performed on 12% gels according to Laemmli (1970). The gels were stained with Coomassie Blue using the standard method.

2.9. Neutralization of the venom lethality

A fixed amount of *Crotalus durissus terrificus* venom and various dilutions of antivenom were incubated at 37 °C for 30 min and injected i.p. in six Swiss-Webster mice. The challenge venom dose corresponded to 5 × LD_{50}. Deaths were recorded for 48 h. Lethality neutralization potency is expressed as μg venom neutralized by 1 mL antivenom, with 50% survival in mice.

2.10. Determination of antivenom antibody titer

The anti-*Crotalus durissus terrificus* antibody titer was determined by ELISA as described by Dong et al. (2003). Briefly, 1:1000 dilutions of samples A to H (Table 2) were transferred to venom-coated microplate wells. After washing, antivenom antibodies were revealed with anti-horse IgG conjugated with peroxidase. The level of antivenom antibodies of each sample was expressed as percentage of positivity respect to the corresponding hyperimmune plasma.

Table 1
Results of two-level factorial design for caprylic acid concentration (4 and 6%), pH (5.3 and 6.3) and temperature (20 and 30 °C).

Sample	Caprylic acid (%)	Temperature (°C)	pH	Turbidity ($A_{600\ nm}$)	Filtration velocity (min/10 ml)	Total protein (g/100 ml)	Albumin (g/100 ml)
1	4	15	5.3	0.070	13	3.3	0.10
1′	4	15	5.3	0.083	12	3.6	0.09
2	4	15	6.3	0.097	15	3.6	0.133
2′	4	15	6.3	0.107	15	3.7	0.161
3	6	15	5.3	0.460	23	3.3	0.116
3′	6	15	5.3	0.412	21	3.7	0.141
4	6	15	6.3	0.216	17	3.6	0.109
4′	6	15	6.3	0.199	18	3.5	0.127
5	4	30	5.3	0.179	6	3.3	0.074
5′	4	30	5.3	0.168	6	3.3	0.094
6	4	30	6.3	0.074	19	3.5	0.082
6′	4	30	6.3	0.098	18	3.7	0.083
7	6	30	5.3	0.460	17	3.7	0.078
7′	6	30	5.3	0.399	18	3.6	0.092
8	6	30	6.3	0.163	12	3.4	0.091
8′	6	30	6.3	0.178	12	3.5	0.107

2.11. Factorial design and statistical analysis of the results

Factorial experiments were designed as described by Box et al. (2005). The effects of temperature, pH and caprylic acid concentration on precipitation responses were studied in two successive 2^3 factorial arrangements (Tables 1 and 2). The responses evaluated included the filtration time (min), the residual albumin content (g/L), turbidity (absorbance at 600 nm), and total protein recovered in the supernatant (g/L). Factorials were subsequently combined in a single set for the regression analysis and fitted to a second-order polynomial equation. The regression analysis was carried out to determine the significance of the effect of the process variables indicated on the responses with the statistical software Statistix 8.0 for Windows. The standard error of the measurements was estimated by using duplicates. For the purpose of the analysis, the data were organized in coded form, as indicated in Table 3. The use of coded process variables allows comparing directly the relative contribution of each independent variable to the prediction of the dependent variable. The higher the positive value of B of a variable, the higher the effect of this variable, and vice versa.

3. Results and discussion

The effect of caprylic acid concentration and the influence of temperature and pH on total protein and albumin in the supernatant, as well as on filtration velocity and turbidity, were firstly studied with a two-level factorial design for three variables (2^3) (Table 1). The values selected for pH (5.3 and 6.3), caprylic acid concentration (4 and 6%) and temperature were around those recommended by Rojas et al. (1994). The results obtained evidenced great differences in filtration velocity and albumin content: while total protein concentration remained similar in all experiments, filtration velocity varied between 6 and 22 min, and albumin content between 0.047 and 0.082 g/100 mL.

ANOVA tests highlighted the significance of all three variables to increase the filtration velocity and to decrease the turbidity of the samples ($p = 0.003$ or less, in both cases) and showed the significance of two- and three-factor interactions. The albumin content and total protein recovery were less affected by the conditions assayed: only caprylic acid concentration displayed a slight effect on the amount of protein recovered ($p = 0.03$, ANOVA tests are displayed as an Appendix). In summary, it is possible to

Table 2
Results of two-level factorial design for caprylic acid concentration (3 and 4%), pH (4.9 and 5.3) and temperature (30 and 37 °C).

Sample	Caprylic acid (%)	Temperature (°C)	pH	Turbidity ($A_{600\ nm}$)	Filtration velocity (min/10 ml)	Total protein (g/100 ml)	Albumin (g/100 ml)
A	4	37	4.9	0.267	6	3.5	0.059
A′	4	37	4.9	0.240	5	3.3	0.065
B	3	30	5.3	0.069	7	3.1	0.061
B′	3	30	5.3	0.072	7	3.4	0.079
C	4	30	4.9	0.095	7	3.7	0.070
C′	4	30	4.9	0.083	6	3.5	0.074
D	3	30	4.9	0.072	3	3.5	0.061
D′	3	30	4.9	0.070	2.5	3.5	0.071
E	4	37	5.3	0.101	2	3.7	0.048
E′	4	37	5.3	0.091	1.5	3.3	0.050
F	3	37	5.3	0.078	2.5	3.5	0.045
F′	3	37	5.3	0.069	3	3.4	0.055
G	3	37	4.9	0.102	5	3.6	0.039
G′	3	37	4.9	0.093	5	3.3	0.043
H	4	30	5.3	0.172	5	3.4	0.074
H′	4	30	5.3	0.162	6	3.5	0.094

Table 3
Range of variables in coded and uncoded form.

Coded values	Uncoded values		
	Caprylic acid (%)	Temperature (°C)	pH
−2	NA	15	NA
−1	3	NA	4.9
0	4	30	5.3
1	NA	37	NA
2	6	NA	6.3

NA: not assayed.

conclude that the increase in temperature and the decrease in caprylic acid concentration and pH bring about an important increase in filtration velocity and a decrease in turbidity, as well as a smaller decrease in albumin content and total protein in the samples.

Therefore, a second round of factorial design experiments was performed, lowering the caprylic acid concentration and pH, and increasing the temperature to 37 °C. Table 2 shows the results obtained, where it is evident that the increase in temperature to 37 °C and the decrease in pH to 4.9 promoted a maximal precipitation of albumin and filtration velocity, with a caprylic acid concentration of only 3%. Lower concentrations of caprylic acid failed to precipitate all the plasma non-IgG proteins. The temperature increase to 37 °C was not deleterious for IgG as judged by the absence of protein aggregation. The two sets of experiments displayed in Table 1 and Table 2 were assembled, the variables expressed in coded form as indicated in Table 3, and analyzed by regression analysis. The combined set of experiments, with 3 variables at 3 levels, was fitted to the experimental data by a second-order polynomial equation, as given below:

$$Yk = Bk0 + \sum Bki \cdot Xi + \sum Bkii \cdot Xi^2 + \sum\sum BkijXi \cdot Xj + ek$$

where: Yk = response: filtration time (min), albumin (g/L), turbidity (absorbance at 600 nm) or total protein (g/L), Xi represents the independent variables: caprylic acid, pH or temperature, Bk0 is the value of fitted response at the central point of the design, and Bki, Bkii, and Bkij are the linear, quadratic and cross- product regression coefficients, respectively (Singh et al., 2007).

Prior to this analysis, the two sets of experiments were subjected to ANOVA, using either the overall data (Table 1 plus Table 2) or two separate blocks (Table 1 or Table 2). As no significant differences in variance were obtained (p values ranging from 0.41 to 1.00 for all responses) the factorial arrangements were combined for the regression analysis.

The second-order polynomial equation adequately represented all responses except that of total protein: for this response, none of the factors were significant, thus indicating that in the range tested, this variable was not relevant for the precipitation process. In all other cases, the R^2 of the proposed model was above 0.90, indicating that the model represented all the experimental data with at least 90% confidence.

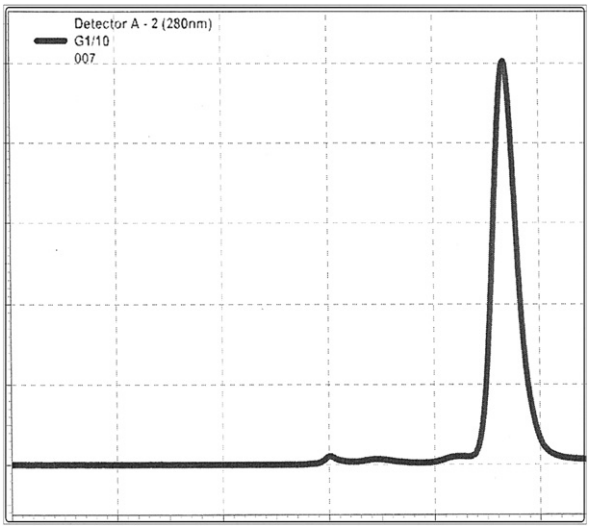

Fig. 1. Size-exclusion HPLC analysis of the final preparation. 2 μl antivenom was loaded to a BioSep S-4000 HPLC column (7.8 × 300 mm; Phenomenex Inc., Torrance, CA, U.S.A.) coupled to Shimadzu Prominence HPLC equipment (Shimadzu Corp., Japan). The column was equilibrated and eluted with 100 mM sodium phosphate buffer, 0.85% NaCl, pH 7.0. The flow rate was 1 ml/min and proteins were detected by their absorbance at 280 nm.

The magnitude of the B and p values indicates the relative contribution of each factor to the response measured; it is therefore evident that, to decrease the filtration time, the three factors (caprylic acid concentration, pH and temperature) were significant ($p \ll 0.05$), and that some of these factors also interacted positively, further decreasing the filtration time. This was particularly true for the cap × pH and pH × T associations.

As regards the remaining albumin, the magnitude of the B and p values also indicated the importance of caprylic acid concentration and temperature ($p \ll 0.05$) and highlighted the importance of the two-factor (pH × T) and three-factor (pH × T × cap) interactions.

As regards turbidity, the decrease in caprylic acid concentration and pH value contributed positively to its decrease, as well as the association between the two factors (cap × pH) and the higher temperature and lower pH (T × pH).

In conclusion, although the optimization conditions for the four responses varied to some extent, the three variables selected were indeed significant for the improvement of the precipitation process. The design chosen also allowed an independent evaluation of second- and third-order interactions; some of them resulted highly significant, thus explaining why the three variables must be taken jointly into account.

A batch of hyperimmune horse plasma was processed under the optimized conditions and the filtrate was concentrated by ultrafiltration, diafiltered against 8.5% sodium chloride and the pH brought to 7.2, and this solution was used for further experiments (Gutiérrez et al., 2005).

Since protein aggregates are largely responsible for unwanted allergic reactions, the presence of protein aggregates was assessed by size-exclusion HPLC. Fig. 1 shows the result obtained, where a large peak

corresponding to IgG and the absence of peaks of higher molecular weight can be seen, thus evidencing the absence of protein aggregates in the preparation.

The albumin concentration of the final preparation was 0.04 g/100 mL and that of the total protein 6.7 g/100 mL. The caprylic acid content was under 0.01 g/100 mL.

Fig. 2 shows the SDS-PAGE of the final preparation in comparison with that of the hyperimmune plasma, where the very low level of albumin is evident. No other proteins are present.

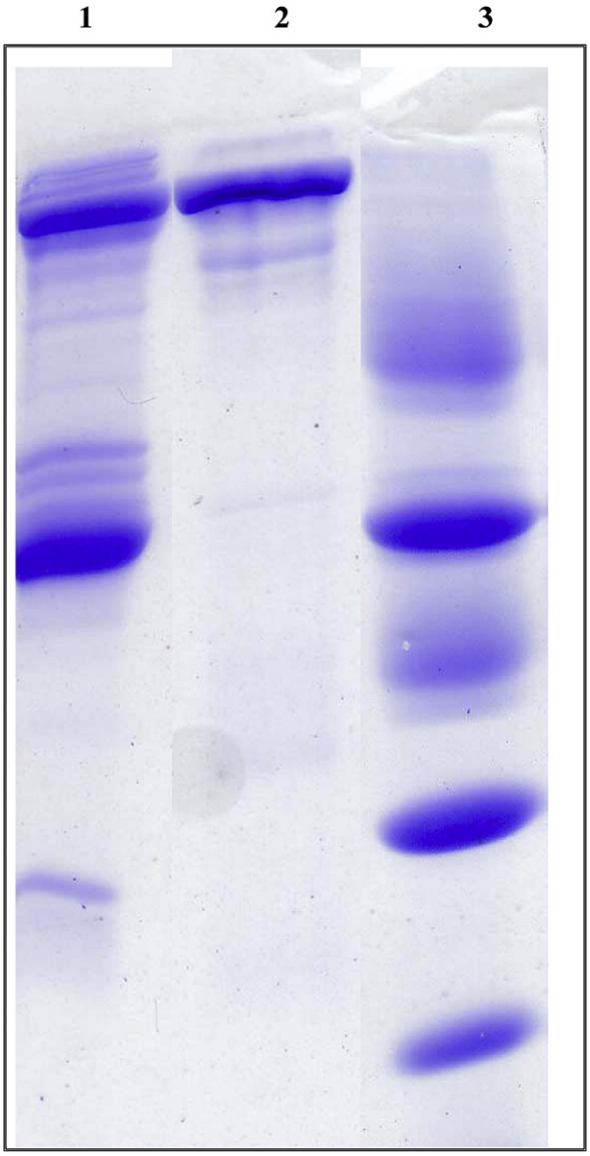

Fig. 2. SDS-PAGE analysis of the final preparation in comparison with the starting material. Two μL samples were loaded to a 12% SDS-PAGE slab. Gels were stained with Coomassie Blue under standard conditions. Molecular weight markers are phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovoalbumin (45 kDa), carbonic anhydrase (31 kDa), trypsin inhibitor (20.1 kDa) and alpha-lactalbumin (14.4 kDa). Samples: 1, hyperimmune plasma; 2, final preparation; 3, molecular weight marker. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Results of the determination of antivenom antibody titer showed that all samples exhibited a similar level of antivenom activity (close to 100%), thus evidencing that it was possible to recover essentially the total antivenom activity present in the horse plasma.

The ability of the antivenom final preparation for lethality neutralization, expressed as μg venom neutralized by 1 mL antivenom was 600 (95% confidence limits = 565–635). Results were similar with plasma of horses immunized with venoms from other snake species.

Finally, if total absence of albumin and caprylic acid in the preparation is desired, they can be adsorbed on an anion-exchange chromatographic matrix at pH 5.5–6.0.

4. Conclusions

By inclusion of temperature among the parameters to optimize the caprylic acid precipitation of hyperimmune horse plasma, an improvement in filtration velocity and remaining albumin was achieved. In addition, since the process requires a lower concentration of caprylic acid, it is more cost-effective.

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Appendix. Supplementary information

Supplementary data related to this article can be found online at doi:10.1016/j.toxicon.2011.10.014.

Conflict of interest statement

None declared.

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