Anion-exchange chromatography in the *batch* mode as a method for purification of equine IgGs from the plasma

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Introduction

Antivenoms are immunoglobulin-derived biopharmaceutical products specifically used to treat the consequences of snakebite envenoming. According to WHO Guidelines for their production, final products should not contain more than 1% of polymer molecules. It has been shown that purification steps involving precipitation of IgGs during downstream processing of plasma might disturb the tertiary structure of immunoglobulins, making them more prone to polymerization. In the same way, contact of IgGs with column proteins or resins during chromatography causes structural changes which can directly lead to aggregation or make IgGs more susceptible to aggregation due to secondary stress. Those are the reasons why we investigated the possibilities of IgG purification by protocols that render IgGs in solution, avoiding their precipitation and temporary binding to matrices of any kind.

Thermally treated hyperimmune horse plasma (HP), the starting material for IgG purification, is a mixture of several proteins. IgG molecules have the higher pI point than most of the other proteins, particularly albumin, main contaminant in plasma purification. Thus, we considered the separation of IgGs on the basis of pI point differences.

AIM:

Driven by the idea to keep IgGs constantly unbound in solution during purification, we investigated the possibility of purifying them by anion-exchange chromatography in the *batch* mode. The influence of different experimental conditions on purity and yield was investigated using full factorial design of experiments.

Materials and methods

Toyopearl SuperQ-650M was used as anionic exchanger. Its behaviour towards human IgG and albumin was determined in the same experimental conditions used for IgG purification from plasma prior to the design of exeperiment.

Design of experiments

Four operating factors: pH, ionic buffer strength, plasma dilution and exchanger binding capacity, each at two levels, were explored to test their influence on the plasma purity and yield in a full factorial experiment. The design of experiment is presented in Table 1.

The protein concentrations in plasma pool and differently treated samples were estimated sepctrophotometrically (Ehresmann method) using following equation:

$(A_{228.5\ nm} - A_{234.5\ nm}) \times 0.3175 = [mg/mL]$.

Appropriate dilution of each sample was independently prepared few times to obtain the mean value of the measured concentrations for further calculation of yield and purity.

One-dimensional **SDS-PAGE** was used to estimate the purity of different supernatant samples. Electrophoresis was done under denaturing conditions at 180 V and 300 mA for one hour.

Purity of IgG (%) in different samples was monitored by size exclusion HPLC. The analysis was done on TSK-Gel G3000SWXL (7.8 x 300 mm) size exclusion column at a flow rate of 0.5 mL/min. The absorbance was monitored at 280 nm using photodiode array detector with phosphate-sulphate running buffer, pH 6.6.

Efficiency of the purification procedure was characterized in each step by **process yield and sample purity**:

Yield was calculated for each step by the following equation: [(*γ* (IgG) x dilution factor) / *γ* (IgG) in starting material] x 100%.

Purity of each intermediate was expressed as: (*γ* (IgG) / *γ* (protein)) * 100%. Purity of highly pure intermediates and final product was additionally proved by HPLC.

Total IgG content (*γ* (IgG)) in all samples was estimated by multiplying the result of SEC-HPLC obtained purity by total protein concentration measrued by Ehresmann method.

The results obtained for purity and yield were analyzed using ANOVA statistical analysis and the individual effect of each tested parameter was presented using Pareto chart.

Table 1. The tested parameters in the experiment were the buffer ionic strength, plasma dilution, pH and exchanger binding capacity.

Exp. run	pH	Ionic buffer strength (mM)	Plasma dilution (x)	Exchanger binding capacity (%)
1	7	35	5	30
2	8	35	5	30
3	7	35	20	30
4	8	35	20	30
5	7	120	5	30
6	8	120	5	30
7	7	120	20	30
8	8	120	20	30
9	7	35	5	60
10	8	35	5	60
11	7	35	20	60
12	8	35	20	60
13	7	120	5	60
14	8	120	5	60
15	7	120	20	60
16	8	120	20	60

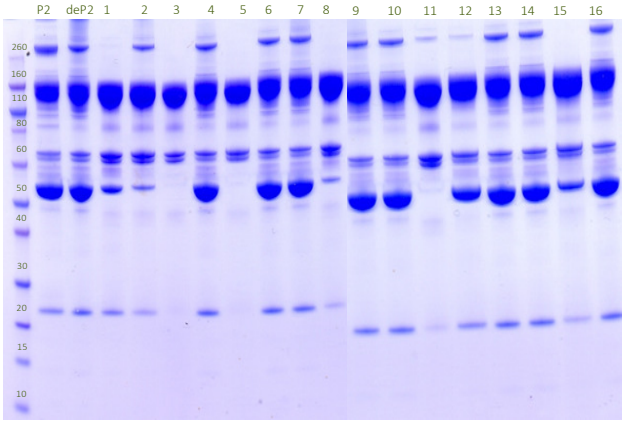

Figure 2. SDS-PAGE gel showing the purification of all samples. The purest samples are 3, 5 and 11, run at 35 mM NaCl and pH 8. Altough their purity is high, the yield in those conditions did not exceed 50%.

Results

Prior to the designed experiment, we determined the binding capacity of Toyopearl SuperQ-650M anion exchanger using pure human albumin and pure human IgG. The determined albuminbinding capacity was 50 mg/mL. In preliminary experiments, we examined the behaviour of pure human albumin and pure human IgG under purification conditions that we wanted to investigate. Albumin was completely bound to the matrix and IgG was almost completely rendered in solution (96%) offering assumption that they would be well separated from plasma.

The statistical analysis of results from 16 experimental runs according to the DoE plan revealed general trend of inversely proportional relationship between thepurity and yield. The ionic buffer strength exhibited the greatest negative effect on the purity of plasma (Figure 2) and the greatest positive effect on the yield (Figure3). The next most influential factor affecting purity was dilution of the plasma and for yield it was pH. The highest IgG purity (83%) was achieved in experiments 3, 5 and 11 (as proved by SDS-PAGE, Figure2) that were done in conditions of pH8 and 35 mM NaCl buffer. However, in these experiments yields did not exceed 35%. In experimental runs where 120 mM NaCl was used, separation was inadequate (Figure 2) which might be connected to the abundance of polymers as determined by SEC-HPLC in the samples where higher NaCl concentration was used(Figure 4).

According to the statistical modeling analysis, the greatest purity and yield, up to 80% and 45%, respectively, could be achieved with the following conditions: 8.63 x diluted plasma, pH 7.05, exchanger volume set at 32% of total binding capacity and 35 mM buffer (Figure 5).

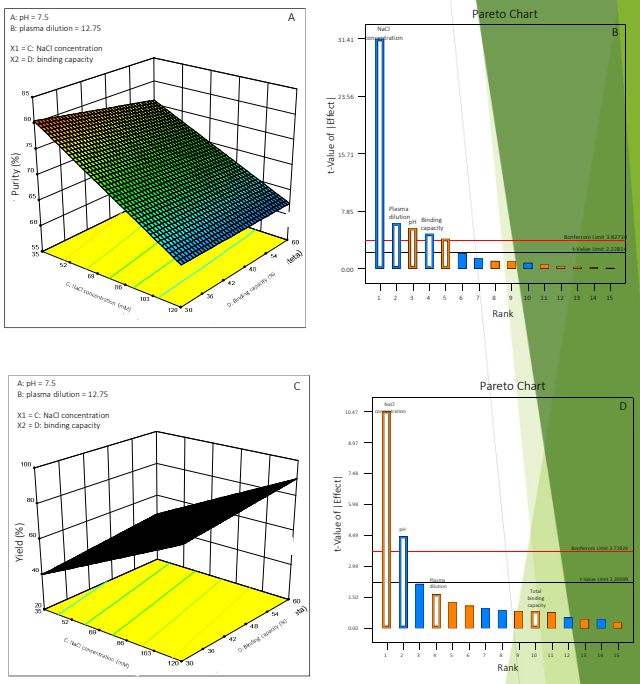

Figure 1. The results of statistical analysis of relative contribution of each tested parameter in plasma purification using Toyopearl 650M-SuperQ anionic exchanger. Purtity is negatively affected by buffer ionic strength (A and B) and yield is positively affected by ionic buffer strength (C and D).

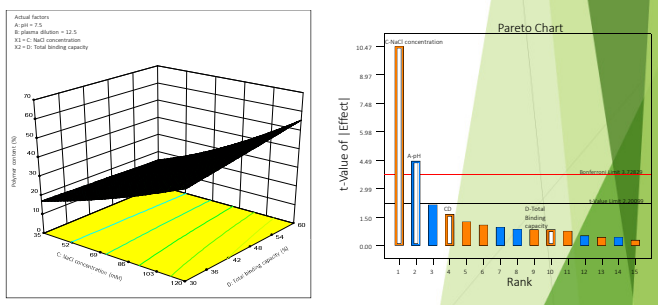

Figure 3. 3-D plot depicts the effect of buffer ionic strength and the binding capacity of anionic exchanger used in experiments on the extent of IgG aggregation.

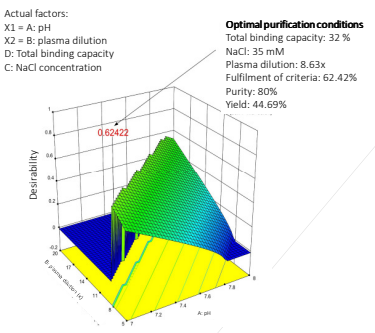

Figure 4. The prediction of optimal reaction parameters which could establish the purity of 80% and yield of 44.69%.

Conclusion

This mode of ion-exchange chromatography can be used to purify IgG up to 80% purity under some experimental conditions, but with huge losses (up to 55%). Such results on purity and yield are inferior to other known purification procedures, so further investigation and optimization needs to be done.

We hypothesize that this incomplete binding of IgG from equine plasma is due to qualitative complexity of plasma content. Equine albumin and equine IgG have close values of pI points. Due to that reason, complete separation from plasma using ion exchanger is harder to achieve. Moreover, albumin and IgG potentially make intermolecular interactions which further lowers the efficiency of their separation. Furthermore, plasma contains the whole set of IgG subtypes that probably have different binding affinites, which is the consequence of their different pI points.

References:

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