A compact and effective procedure for antivenom downstream processing

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Introduction

Antivenoms are the only specific therapeutics effective for counteracting post-snakebite pathophysiological manifestations. The starting material for their production is hyperimmune animal plasma, mostly equine or ovine. There are several well-established plasma purification protocols that are routinely implemented in the antivenom commercial scale production. However, the development of compact low-cost protocols yielding safe and efficient products is still of great concern. The optimization of downstream manufacturing protocols is aimed at fulfilling several factors: increase in the final product yield, maximizing the final product purity, lowering manufacturing expenses, shortening processing time, and respecting all the regulatory demands for antivenom manufacturing.

Here, we present the development of a compact and effective procedure for antivenom downstream processing resulting in pure $F(ab')_2$ fragments as the final product. The ultimate goal was to include and combine only steps in which active drug (IgG or $F(ab')_2$) would be constantly dissolved (not precipitated, not bound to the column) throughout the whole process.

Methods

Total IgG content (γ(IgG)) in all samples throughout the purification procedure was estimated by ELISA determining of venom-specific IgG fraction in which total IgG isolated from venom-specific hyperimmune plasma by protein A chromatography served as a standard. Considering that different plasma pools contain different content of venom-specific IgG, correction of ELISA results was included. It was done based on the shift of the ELISA result from total protein concentration obtained for 100 % pure IgG sample isolated from particular plasma pool. The method is precise, accurate, specific and correlates with *in vivo* neutralizing potency results (Table 1).

Total $F(ab')_2$ content ($\gamma(F(ab')_2)$) was estimated by analogous ELISA method, using $F(ab')_2$ purified from European viper venom antiserum (Zagreb antivenom, Institute of Immunology, Inc., Croatia) by diafiltration as a standard.

Total protein concentration (γ(protein)) was estimated spectrophotometrically according to Ehresmann *et al*. (1973).

Purity of IgG/$F(ab)_2$ (%) in all purification steps 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.

Neutralising capacity of hyperimmune plasma and pure IgG sample was determined by *in vivo* assay in mice, according to Ph. Eur. (2017) and WHO guidelines (2010), with experimental details in Kurtovic *et al.* (2012). *R*-value (expressing the number of LD_{50} doses that could be neutralized by 1 mL of a sample) was used as a measure of protective efficacy.

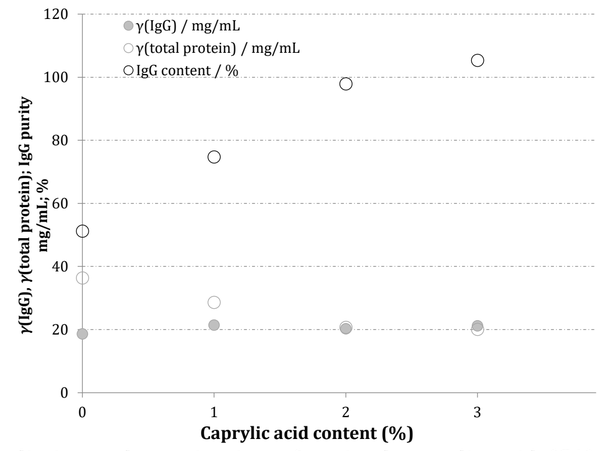

Figure 2. Preliminary determination of optimal caprylic acid (CA) concentration for precipitation step of the purification protocol. Both 2% and 3% CA solutions give purity of 100%.

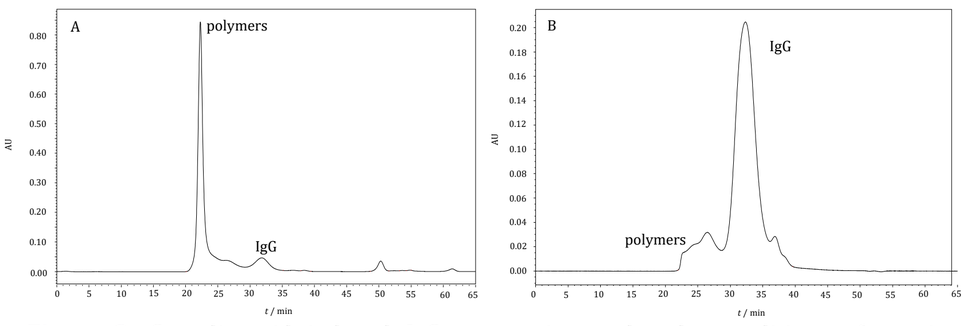

Figure 3. Caprylic acid induced IgG aggregation under the conditions of pepsin digestion (A). Removal of caprylic acid by diafiltration drastically reduces IgG aggregation (B).

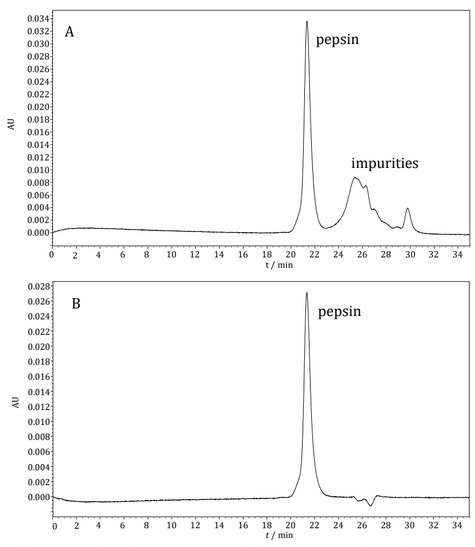

Figure 4. Commercially available pepsin (A) is completely retained on CIM QA under conditions used in anion-exchange chromatography for final polishing of $F(ab')_2$ and eluted from the disk with 100% yield.

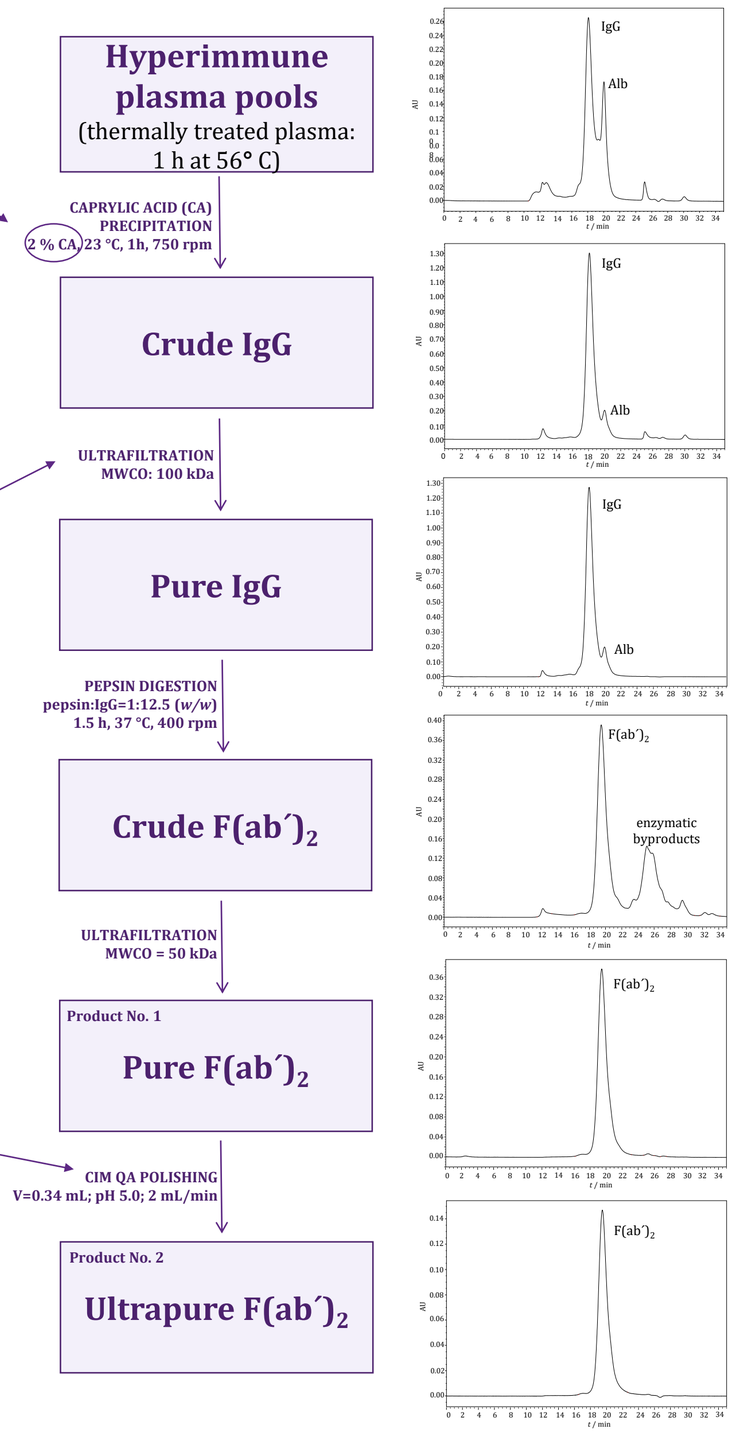

Figure 1. Schematic presentation of the developed plasma purification protocol with corresponding chromatograms of representative samples in each purification step. Three major steps are: caprylic acid (CA) precipitation, pepsin digestion of IgG molecules and final CIM QA polishing. Between the steps, plasma was filtered through PVDF filter and ultrafiltered after steps 2 and 4. Two exit points of the procedure are possible, before and after final CIM QA polishing, both yielding $F(ab')_2$ as the final product..

Table 1. Comparison of plasma and pure IgG neutralisation potency determined *in vivo.* Results are given as +/- SE.

	plasma	pure IgG
R / LD_{50} mL^{-1}	48 ± 11	49 ± 3
γ (IgG) / mg mL^{-1}	34 ± 5	35 ± 2
specific activity / LD_{50} mL^{-1}	1.4	1.4

Table 2. Yields and purities of intermediates and final product obtained by developed downstream processing protocol. +/- indicates 95% CI.

	IgG / $F(ab')_2$ purity (%)	IgG / $F(ab')_2$ yield (%)
crude IgG	81.3 ± 2.1 (n=6)	97.3 ± 0.7 (n=6)
pure IgG	89.0 ± 1.1 (n=5)	98.7 ± 5.0 (n=7)
crude $F(ab')_2$	56.3 ± 6.9 (n=7)	90.3 ± 8.6 (n=7)
pure $F(ab')_2$	100.3 ± 5.7 (n=7)	84.2 ± 1.2 (n=6)
ultrapure $F(ab')_2$	100.0 ± 2.6 (n=6)	82.3 ± 5.5 (n=8)

Results

Downstream processing of venom-specific horse plasma consists of the following steps (Figure 1):

1. caprylic acid precipitation (for removal of non-immunoglobulin proteins); 2% caprylic acid was chosen as the minimal quantity removing almost all unwanted proteins (Figure 2);
2. diafiltration of crude IgG (for removal of caprylic acid); caprylic acid strongly triggers IgG aggregation under conditions of the subsequent peptic digestion (Figure 3);
3. peptic digestion (for $F(ab')_2$ fragment generation);
4. diafiltration (for removal of small by-products of enzymatic digestion);
5. anion-exchange chromatography (for complete removal of pepsin); traces of pepsin in final antivenom preparation might cause reduced stability; conditions chosen for CIM QA polishing efficiently remove pepsin from the sample (Figure 4).

The procedure gives ultra pure $F(ab')_2$ fragments of 100 ± 2.6 % purity, in high yield (82.3 ± 5.5 %) and with no aggregates (Figure 1, Table 2.). The procedure can be stopped before final polishing by anion-exchange chromatography, since purity of pure $F(ab')_2$ is also high (100.3 ± 5.7%) (Table 2). The quality of both pure and ultrapure $F(ab')_2$ products fulfills regulatory demands.

In addition, *in vitro* method was developed enabling precise estimation of purity and yield throughout the purification procedure, thus eliminating the need of animal use for process monitoring.

References:

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