Simple and effective approach for antivenom manufacturing

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INTRODUCTION

Antibody-based therapeutics play an important role in specific treatment of some medical emergencies. One of the most widely used representatives are antivenoms obtained from hyperimmune animal plasma, mostly equine or ovine, as the only specific therapeutics irreplaceable for counteracting post-snakebite pathophysiological manifestations. Although there are various well established refinement processing strategies that have been implemented into their commercial scale production, optimisation of high yielding and low-cost manufacturing process generating safe, effective and available immunotherapeutic is still of great concern.

AIM

We present development of a simple, feasible and economically viable purification strategy for preparation of equine plasma-derived antivenom based on IgGs or F(ab')$_2$ fragments. Our goal was to design simple and easily scalable sequence of purification steps in which desired IgG molecules or their fragments would be kept in solution thoroughly, as a precautionary measure against possible degradation/aggregation of the active principle due to precipitation or binding to chromatographic supports. At the same time, we aimed to meet two key requirements which indicate successfulnes of the developed procedure - preservation of the process yield at the highest possible level and fulfilment of the regulatory requirements concerning final product purity.

MATERIALS AND METHODS

Total IgG content (γ(IgG)) was estimated by ELISA determining the venom-specific IgGs in which total IgG isolated from the venom-specific hyperimmune plasma by protein A chromatography served as a standard. Considering that different plasma pools vary in content of venom-specific IgGs, 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* neutralising potency results (Table 1).

Total F(ab')$_2$ content (γ(F(ab')$_2$)) was estimated by analogous ELISA, using F(ab')$_2$ purified from European viper venom antiserum (Zagreb antivenom, Institute of Immunology, Inc., Croatia) by ultrafiltration as a standard.
Total protein concentration (γ(protein)) was estimated spectrophotometrically (Ehresmann *et al.*, 1973).
Purity of IgG/F(ab')$_2$ (%) in all purification steps was monitored by SEC HPLC on TSK-Gel G3000SW$_{XL}$ column (7.8 x 300 mm) with 0.1 M phosphate-sulphate running buffer, pH = 6.6, at a flow rate of 0.5 mL/min.

Efficiency of the purification procedure was characterised in each step by process yield and sample purity. Yield was calculated 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% and additionally proved by SEC HPLC.

Neutralising capacity of hyperimmune plasma and pure IgG sample was determined by *in vivo* assay in mice (Ph. Eur., 2017). R-value (the number of LD_{50} doses that can be neutralised by 1 mL of a sample) was used as a measure of protective efficacy.

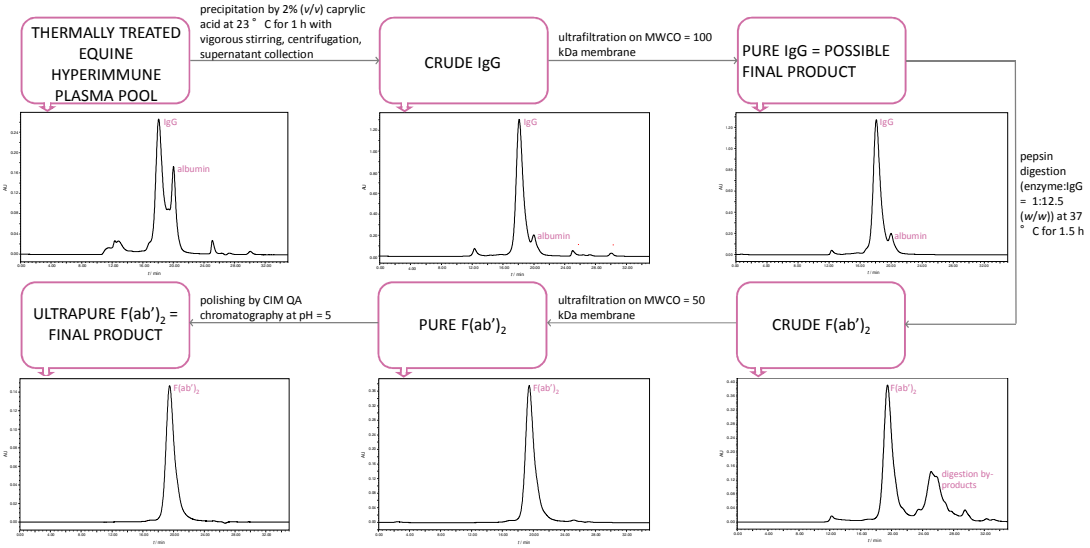

Figure 1. Schematic presentation of the purification protocol with corresponding chromatograms of representative samples in each step. Three major steps are: caprylic acid precipitation, pepsin digestion of IgG molecules and CIM QA polishing. Two exit points of the procedure are possible, after ultrafiltration of crude IgG preparation obtained by caprylic acid precipitation and after CIM QA polishing, yielding IgG or F(ab')$_2$ as the final product, respectively.

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

	PLASMA POOL	PURE IgG
R / [LD_{50} mL^{-1}]	48 ± 11	49 ± 3
γ(IgG) / [mg mL^{-1}]	34 ± 5	35 ± 2
Specific activity / [LD_{50} mg^{-1}]	1.4	1.4

Table 2. Yields and purities of the intermediates and the final product obtained by developed downstream processing protocol. Results are given as +/- 95% CI.

	IgG / F(ab')$_2$ PURITY [%]	IgG / F(ab')$_2$ YIELD [%]
Plasma	46.3	n.a.
Thermally treated plasma	47.8	93.4
Crude IgG	81.3 ± 2.2 (n = 6)	89.6 ± 3.6 (n = 8)
Pure IgG	89.0 ± 1.4 (n = 5)	91.5 ± 2.0 (n = 7)
Crude F(ab')$_2$	56.3 ± 6.4 (n = 7)	84.1 ± 10.5 (n = 7)
Pure F(ab)$_2$	100.3 ± 5.2 (n = 7)	78.6 ± 3.0 (n = 6)
Ultrapure F(ab')$_2$	100.0 ± 2.8 (n = 6)	77.0 ± 2.3 (n = 6)

RESULTS

Fractionation of the venom-specific plasma was effectively performed by means of only few purification steps, as shown in Figure 1.
Unwanted proteins, mostly albumin, were precipitated by 2% caprylic acid, leaving IgGs in solution.
Next, precipitating agent was removed from IgG-enriched fraction by ultrafiltration. Namely, caprylic acid provokes aggregation of IgG molecules under conditions favoring their efficient conversion to F(ab')$_2$ fragments, subsequently performed by means of pepsin digestion.
After ultrafiltration for removal of low molecular weight by-products of enzymatic cleavage, the obtained F(ab')$_2$ preparation was additionally polished by ion-exchange chromatography on CIM QA disk, achieving preparation of the active principle free from pepsin and other residual impurities, and consequently, drug of improved stability.

Developed procedure gives completely pure and aggregates-free F(ab')$_2$-based product of over 75% yield, as monitored by several *in vitro* methods (Table 2, Figure 1). IgG fraction, an intermediate produced by combination of precipitation and ultrafiltration with almost no losses, also complies with regulatory requirements concerning impurity content. Thus, designed processing strategy could be utilised as a platform for production of both types of immunotherapeutics.

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.

EDQM (2017) Viper Venom antiserum, European (04/2008:0145). Europea Pharmacapoeia 9.0, 1119-1120.
Ehresmann et al. (1973) Anal. Biochem. 54, 454–463.

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