# A NOVEL METHOD FOR PRECISE QUANTIFICATION OF IGG IN HYPERIMMUNE HORSE PLASMA

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## INTRODUCTION

The hyperimmune horse plasma (HHP), prepared by an active immunisation of horses with an antigen of interest, is a starting material for antitoxin production (tetanus antitoxin, rabies antitoxin, antivenom). Antitoxins are pure horse immunoglobulins or their fragments, with the ability to neutralise respective antigen (pathogen, toxin, venom). Precise determination of the IgG quantity in the starting plasma is a prerequisite for accurate estimation of process efficiency by *in vitro* methods. The currently used methods, for both human and animal plasma, are SDS-PAGE with densitometric analysis of electropherograms or the gel filtration chromatography with peak area analysis[1,2] (Figure 1). These assays determine the ratio of immunoglobulin/ non-immunoglobulin proteins.
However, these methods are not accurate so methods that measure antibody activity have to be used for precise production process yields monitoring. In the case of antivenoms, these methods are *in vivo* methods in mice.

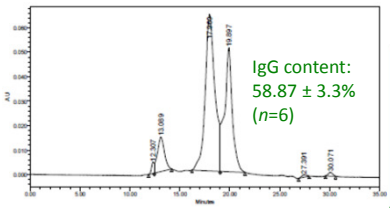


**Figure 1.** IgG content in HHP determined by HPLC size exclusion chromatography with peak area analysis.

### AIM

In order to completely avoid the usage of animals in yield calculation during antivenom downstream processing, we aimed to develop *in vitro* method for precise and accurate measurement of IgG content in HHP.

## MATERIALS AND METHODS

Crude venom of Vipera ammodytes ammodytes L. (Vaa venom) was collected by milking snakes kept at the Institute of Immunology Inc., Croatia, air dried at ambient temperature and stored in the dark at 4 °C until use. Vaa venom-specific hyperimmune horse plasma was obtained from the Institute of Immunology Inc., Croatia. Mice, both sex 18-20 g, used for in vivo assays were bred at the Institute of Immunology. Procedures, handling and animal work were in accordance to the Croatian Law on Animal Welfare (2010) which complies with EC Directive 2010/63/EU.

**Internal reference IgG preparation**

Caprylic acid was added dropwise to 1 mL of defibrinated equine venom-specific plasma pool. Its final concentration in reaction mixture two fold diluted with saline was 2% (*V*/*V*). Precipitation occurred by vigorous stirring at 23 °C for 1 h. After centrifugation at 2800 x g for 45 min, supernatant (containing the IgG fraction) was collected, filtered through a 5 µm filter and diafiltered into 150 mmol/dm$^3$ NaCl using 100 kDa molecular weight cut-off membrane. IgG preparation was analysed by ELISA and SEC HPLC as described, aliquoted and stored at -20 °C.

**Protein concentration estimation**

The protein concentrations in hyperimmune horse plasma and in samples from all purification steps were estimated spectrophotometrically (Ehresmann's method) using following equation: (*A*228.5 nm – *A*234.5 nm) × 0.3175 = [mg mL$^{-1}$]. Appropriate dilution of each sample was independently prepared three times to obtain the mean value of the measured concentrations for further calculation of yield and purity.

**SEC HPLC (size - exclusion chomatography)**

The IgG content in HHP and the purity of internal reference IgG was determined by HPLC size exclusion chromatography using TSK-Gel G3000SWXL (7.8 x 300 mm) column. Analyses were run at a flow rate of 0.5 mL min$^{-1}$ at room temperature and the eluate was monitored at 280 nm. The running buffer was 0.1 M phosphate sulphate buffer, pH 6.6. Standard proteins used for molecular weight determination were tyroglobulin (Mr 669000), γ-globulin (Mr 150000), ovalbumin (Mr 43000), and ribonuclease A (Mr 13700).

**ELISA**

ELISA for detection of venom - specific antibodies in samples from hyperimmune horse plasma processing was performed by coating microtiter plate with 100 µL/well of venom coating solution (1mg/mL) in 50 mM carbonate buffer, pH 9.6, and left overnight at room temperature (RT). After blocking with 0.5 % (*w*/*V*) BSA in PBS/T (0.05% (*V*/*V*) Tween 20 in PBS) buffer at 37 °C for 2 h, the plasma or samples from each purification step were added in two-fold serial dilutions in duplicates and left overnight at RT. The antibodies isolated from hyperimmune plasma by protein A affinity chromatography were used as a standard. In the subsequent steps, incubation with HRP-anti-horse F(ab')$_2$ (25 000-fold diluted) at 37 °C for 2 h occurred, followed by the addition of OPD (0.6 mg/ml solution) in citrate phosphate buffer, pH 5.0. After 30 min of incubation in the dark, the enzymatic reaction was stopped with 1 M $H_2SO_4$ and the absorbance at 492 nm was measured.

**$LD_{50}$ and $ED_{50}$ test**

The potential of plasma and pure IgG samples to neutralise the venom's lethal toxicity was determined by lethal toxicity neutralisation assay in mice, as follows. Two-fold serial dilutions of samples were preincubated with equal amounts of the venom solution containing two median lethal doses ($LD_{50}$, the amount of dry venom (in µg) causing the death in half of the mice population used). The immunoprecipitates were removed by centrifugation and clear supernatants *i.v.* administered to groups of four mice. Deaths were recorded 48 hours later. For each sample the median effective dose ($ED_{50}$, the amount of undiluted serum capable of neutralising the venom's lethal effect in 50 % of the animals) was determined. The lethal toxicity neutralisation potency (*R*) was expressed as the number of $LD_{50}$ venom doses that can be neutralised by 1 mL of undiluted sample and calculated by the equation $R=(Tv-1)/ED_{50}$ where Tv represents the number of $LD_{50}$ inoculated per mouse. *R* value was used as a measure of the protective efficacy of each sample.

## CONCLUSION

We successfully developed a novel method for quantification of IgG in hyperimmune horse plasma. The method was proved as precise, accurate, and specific, and fulfills regulatory requirements. It revelaled the IgG content in HHP is significantly lower (46 %) than estimated by currently used methods (60 %).

## RESULTS

**The principle of our approach** was to measure IgG population specific for venom in the HHP, that would serve as a predictor of the whole IgG population.

An ELISA has been developed in which venom coated wells are incubated with HHP or IgG standard in appropriate dilutions. Venom specific IgGs bound to the wells are subsequently detected and visualised by anti-horse polyclonal antibodies conjugated to horse-radish peroxidase. IgG quantification is performed by comparing the response of HHP plasma sample to response of affinity purified IgG fraction from HHP that served as a standard of known concentration (measured by total protein determination).
Such method would give precise results, if IgG fraction would be of equal composition in all samples and IgG standard as well. However, this is not the case.

We had to face the following **OBSTACLES**: → **PROBLEM**

1. The IgG fraction of a plasma is a heterogenous population of immunoglobulins that differ in class, subclass, or specificity (Figure 2).
2. In addition, each plasma sample has a different distribution of these different immunoglobulin molecules (dominantly as a consequence of different response of horses in hyperimmunization procedure).
3. Protein A affinity purified IgG fraction from horse hyperimmune plasma (used as a standard for quantification) has lower amount of IgGa, IgGc and IgGT classes, due to their low binding to protein A[3].
4. Any change (difference) in class or subclass distribution in a sample can affect the ELISA measurement, in which detection is made by a polyclonal anti-horse IgG reagent.

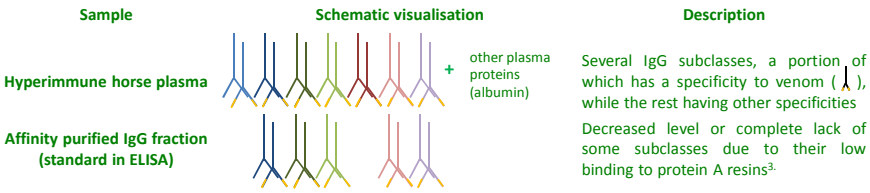


**Figure 2.** Shematic presentation of differences in composition between IgG fraction in hyperimmune horse plasma and IgG fraction isolated from the same sample by protein A affinity chromatography

## SOLUTION:
## introduction of internal reference for each plasma sample

We found the simple, fast and cheap protocol for total IgG isolation from plasma, which **does not affect its original composition**, as proved by antibody functionality tested *in vivo* (Table 1). Specific neutralization efficiency ($LD_{50}$/mg IgG) was equal (1.39) for plasma and its pure IgG fraction, in contrast to IgG (denoted as eIgG) isolated from the same plasma pool by protein A affinity chromatography (0.85). The protocol is based on caprylic acid precipitation of non-IgG proteins, while leaving IgG in solution. The procedure yields almost 100% pure IgG fraction (Figure 3). Its IgG content was quantified by protein quantification. Well characterized total IgG fraction from each plasma pool, with precisely determined concentration, served as an internal standard, and was used for appropriate correction of ELISA results for the respective plasma pool. Analysis of two HHP pools (Table 2) by the novel method illustrates perfectly the influence of different IgG fraction composition on the outcome of the ELISA results.
IgG concentration was 56.41 mg mL$^{-1}$ for P1, and 18.27 mg mL$^{-1}$ for P2 pool, while both had similar total protein concentration (75 and 67 mg mL$^{-1}$, respectively) suggesting that crude ELISA results are not accurate. After correction of both results according to thier respective internal references, IgG concentrations were 35.53 and 31.15 mg mL$^{-1}$, respectively. In other words, HHP contains 46 % of IgG, which is significantly lower in comparison to cca 60% obtained by HPLC analysis (Figure 1) and reported so far[4]. The novel assay was validated, and proved to fulfill regulatory requirements for precision, accuracy, linearity and specificity.

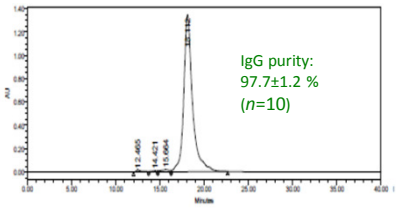


**Figure 3.** Internal reference IgG sample purity estimation by HPLC size exclusion chromatography.

**Table 2.** IgG quantification by the novel ELISA method with correction of results according to internal reference IgG

| | P1 | P2 |
|---|---|---|
| ***c*(IgG) / mg mL$^{-1}$** | | |
| crude ELISA result | 56.41 ± 1.5 (*n*=26) | 18.27 ± 1.3 (*n*=27) |
| ELISA result corrected according to internal reference | **35.53 ± 1.9 (*n*=26)** | **31.15 ± 2.3 (*n*=27)** |
| ***c*(protein) / mg mL$^{-1}$** | **75.05 ± 1.8 (*n*=17)** | **66.80 ± 2.4 (*n*=12)** |
| **IgG content (%)** | **47.3 %** | **46.6 %** |

**Table 1**. Equal specific neutralization efficiency of IgGs in plasma and of internal reference IgG sample indicates equal compostion of IgG pool.

| | plasma | eIgG | internal reference IgG |
|---|---|---|---|
| **[1]*R*/ $LD_{50}$ mL$^{-1}$** | 48.0 ± 11.1 (*n*=3) | 48.9 ± 7.5 (*n*=5) | 48.9 ± 2.7 (*n*=3) |
| **[2]γ(IgG)/ mg mL$^{-1}$** | 34.4 ± 4.5 (*n*=5) | 57.2 | 35.3 ± 2.2 (*n*=5) |
| **[3]specific activity/ $LD_{50}$ mg$^{-1}$** | **1.39** | 0.85 | **1.38** |

[1] *determined by in vivo neutralisation potency assay*
[2] *determined by ELISA with correction of results*
[3] *calculated as R / γ(IgG)*

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**Acknowledgements:**
This work was fully financially supported by Croatian Science Foundation (IP-2014-09-4915). Any opinions, findings or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of Croatian Science Foundation.