UDC 571.24+615.074

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# PHYSICO-CHEMICAL AND BIOLOGICAL PROPERTIES OF SOLVENT/DETERGENT TREATED IMMUNOGLOBUIN G PREPARATIONS

*Protein composition homogeneity, fraction composition, molecular weight composition, anticomplementary activity, anti-A and anti-B haemagglutinins, specific activity, and pyrogens were studied in intramuscular and intravenous immunoglobulin G preparations, treated and non-treated with solvent/detergent mixture for virus inactivation. Physico-chemical and biological properties retention is shown. Quality of immunoglobulin preparations was improved due to purification by the methods of anion- and cation- exchange preparative low pressure liquid chromatography.*

*K e y w o r d s: immunoglobulin preparations, virus inactivation, solvent/detergent, chromatography purification.*

Immunoglobulins as antibodies provide highly specific protection against attacks in an organism. The main function of immunoglobulins is the antigen binding, neutralizing of bacterial toxins, viruses, voiding their penetration into cells. These molecules provide antiviral and antimicrobial protection. Immunoglobulin functions are related to Fc-region of the molecule, which, while binding to reticuloendothelial system cells and complement components, helps in the invader opsonization, phagocytosis activation, and lysis.

Usage of immunoglobulin preparations for intramuscular and intravenous injection is considered to be a priority area in immunotherapy and immunoprophylactics of infectious diseases [2, 6, 11, 12, 19, 20].

The viral safety is the main problem in immunoglobulin preparations manufacturing from donor blood plasma. The production process, which must include virus inactivation and removal steps with proved efficiency, has to play the key role in the safety insurance. Many virus inactivation techniques may affect physico-chemical properties, stability, specific activity, and half-life of immunoglobulin in the organism, resulting in the decline of drug efficiency [1]. Virus inactivation method must provide the assured safety on the one hand, and retention of native structure and functions on the other hand.

This is why the investigation of physico-chemical and biological properties of solvent/detergent (S/D) treated immunoglobulins appears to be so important.

**Materials and methods**

Three immunoglobulin G medications, produced by the Cohn (modification B) alcohol precipitation method [18] manufactured by JSC *Biopharma*, were studied: Human normal immunoglobulin for intramuscular administration 10%, Venoimmune 5 % (pepsin treated intravenous immunoglobulin), and Bioven Mono 5 % (unmodified intravenous immunoglobulin).

S/D virus inactivation and chromatography purification was performed according to [8,9].

S/D treated batches were compared to non-treated ones of the same preparations. The next quantities of S/D treated and non-treated full-scale batches, respectively, were used in the study: Human normal immunoglobulin for intramuscular injection 10 % – 60 and 28; Venoimmune 5 % – 19 and 22; Bioven Mono 5 % – 11 and 7.

Protein composition homogeneity (electrophoretic purity) was determined by zonal electrophoresis method [4, 5]. Protein fractions identification was made by comparison with human blood plasma electrophoregrams. Electrophoregrams were processed using TotaLab V.2.01 Software (GE Healthcare, USA).

Fraction composition was determined by immunoelectrophoresis method [4, 15]. The position and number of precipitation bands were compared to those of the human blood serum.

Monomer, dimer, aggregates, and fragments determination (molecular-weight composition) was performed by high performance size-exclusion chromatography [4]. Chromatography column TSKgel G3000 $SW^{xl}$ 7.5x600 mm (Supelco 805103) and reference preparation (industry standard) OCO 42-28-301–06 П (Tarasevich SISC, Moscow) were used.

Anticomplementary activity (ACA) was determined in the complement binding reaction [14].

Anti-A and anti-B haemagglutinins were determined according to [4].

Specific antibodies activity to antistaphylolysin was determined by neutralization reaction [13, 15], to versatile virus – by passive haemagglutination reaction [15], to Hbs-antigen – by Elisa method according to commercial kit's manual [15].

Pyrogens were determined according to [4].

Student's method for pairwise-independent variants was used [16], to estimate the statistical significance.

**Results and Discussion**

Development of virus inactivation technique for immunoglobulin preparations is complicated due to the high lability of the protein molecule, which can be denaturated under certain physico-chemical conditions. This may result in changes of the functional behavior.

The molecular-weight composition is one of the most important criteria of immunoglobulin stability. The presence of polymers and aggregates in immunoglobulin preparations can lead to hypotensive effect when administered intravenously, this factor ensures harmlessness and tolerability of the preparation. Non-specific complement activation caused by aggregates is proved to be a reason of adverse reactions during intravenous immunoglobulin administration [3, 7, 22]. To investigate aggregates and fragments behavior during chromatography purification the venoimmune preparation solution was purified on the small scale. High aggregates concentration was intentionally induced by incubation in 1 M NaCl solution. The fragments (20–25 %) are generated by pepsin treatment during manufacturing process. The molecular-weight composition was determined before and after each purification step. Chromatograms of some fractions are presented in Fig. 1. Aggregates are removed from the solution at the 1st purification step (anion-exchange chromatography on the diethylaminoethyl adsorbent). Monomer, dimer and fragments do not interact with the adsorbent in the chosen conditions (Table 1). The aggregates are retained, possibly losing their solubility in the buffer used. No significant changes were observed in the fragments composition. All S/D treated preparations studied complied requirements for molecular-weight composition even on the 6th month after shelf-life period expiration.

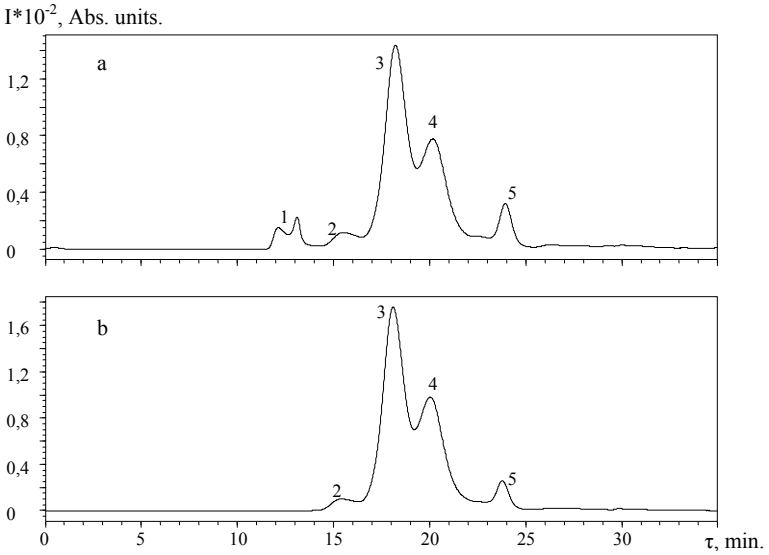


**Fig. 1. Chromatograms of small-scale Venoimmune sample before (a) and after chromatographic purification (b).** 1 – polymers and aggregates, 2 – immunoglobulin G dimmer, 3 - monomer, 4 – fragments $Fab_1$, 5 – fragments $Fab_2$.

ACA is one of the most critical values affecting tolerance to intravenous immunoglobulin administration [21]. According to requirements [4, 17], acceptable limit of complement binding activity is not more than one haemolytic unit $CH_{50}$ per one milligram of protein. No significant change was observed in ACA after aggregates removal by chromatography (Table 1).

For immunoglobulin preparations determination of ballast plasma proteins by cellulose acetate electrophoresis is mandatory. A significant difference was determined: S/D treated batches had a lower amount of impurities as compared to non-treated ones (Table 1). Examples of electrophoregrams and software data processing are presented in Fig. 2.

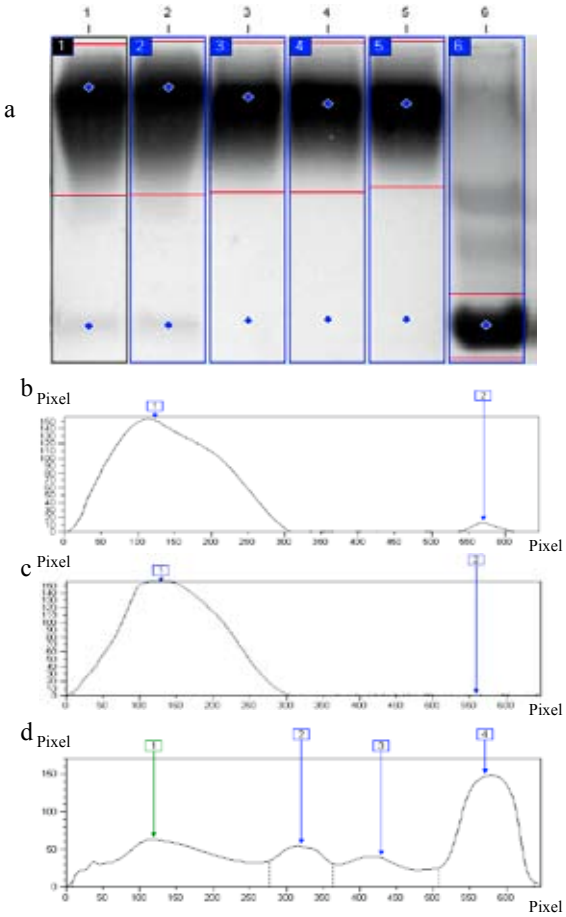


**Fig. 2. Electrophoregrams (a) and densitograms (b,c,d) of immunoglobulin samples – non-treated (a: lanes 1,2 and b) and treated (a: lanes 3, 4, 5 and c); normal human plasma (a: lane 6 and d).**
1 – immunoglobulin G; 2 – β-globulins; 3 – α-globulins; 4 – albumin.

Foreign proteins presence is undesirable, since every protein from plasma pool acts as an antigen for a separate organism. Besides the main fraction, immunoglobulins may contain different quantities of ballast proteins – albumin, plasminogen, immunoglobulins A and M, which may cause adverse reactions. For example, patients with selective immunoglobulin A deficiency produce anti-IgA antibodies. Such patients must receive immunoglobulin G preparations with low immunoglobulin A concentration in order to avoid adverse reactions [3, 6]. A number of commercial immunoglobulin G preparations from the world leading companies, having the same quality, differ in price, because they contain different immunoglobulin A trace concentrations [3]. Obtained data testify to additional removal of contaminating proteins, including immunoglobulins A and M, during chromatographic purification (Table 1). Immunoelectrophoregrams of S/D treated and non-treated immunoglobulin preparations are presented in Fig. 3. Most studied S/D treated samples show a higher homogeneity of fraction composition as compared to non-treated ones. Only 1 or 2 precipitation bands are visible.

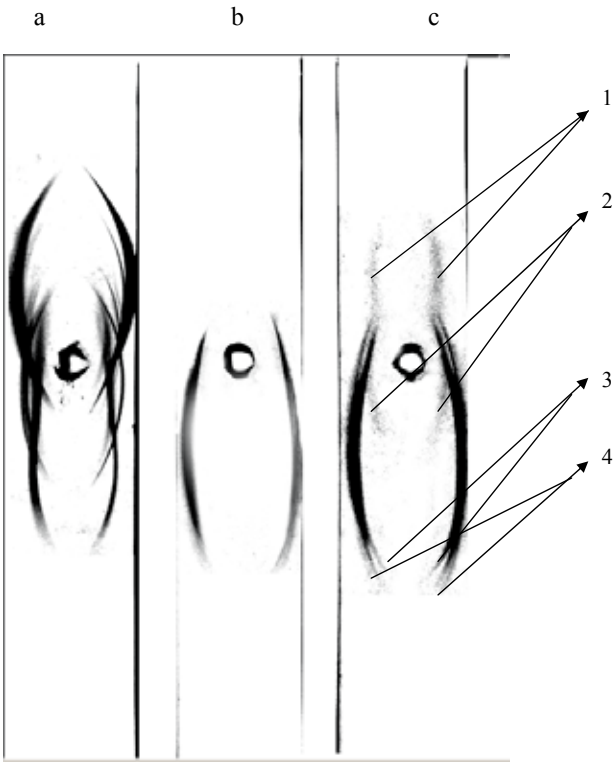


**Fig. 3. Immunoelectrophoregrams of the following samples:** a – human plasma serum, b – treated immunoglobulin sample, c – non-treated immunoglobulin sample. 1 – albumin, 2 – immunoglobulin M, 3 – immunoglobulin A, 4 – immunoglobulin G.

An important item to study, even after an insignificant change of the manufacturing process, is retention of biological activity of immunoglobulin, which determines clinical efficiency of the preparation. For the proper assessment of molecule functional condition, specific activity determination of no less than two types of antigens (one virus and one bacterial) should be carried out. According to normative documentation [17], antibodies to antistaphylolysin were determined.

Neutralization of viruses with antibodies is a mechanism of hepatitis B transmission risk reduction. That is why, according to international regulations, all immunoglobulin preparations (normal and specific) should be assayed for hepatitis B surface antigen antibodies (anti-HbsAg). According to actual Ukrainian requirements [4], anti-HbsAg activity for immunoglobulin preparations should be no less than 0.5 IU/g. WHO recommends this value to be no less than 1 IU/g [21].

A comparison of antibodies activity shows no statistically significant difference for the treated and non-treated immunoglobulin batches (Table 1).

Table 1

**Arithmetic average values of immunoglobulin medications quality control results**

| Item | Tests results average values | | | | | | | | | Excerptings difference reliability. Confidence coefficient 0.95<b<0.99 | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Human normal immunoglobulin 10 % | | | Bioven Mono 5 % | | | Venoimmune 5 % | | | Human normal immunoglobulin 10 % | Bioven Mono 5 % | Venoimmune 5 % |
| | Permissible limit | S/D non-treated | S/D treated | Permissible limit | S/D non-treated | S/D treated | Permissible limit | S/D non-treated | S/D treated | | | |
| Aggregates, % | < 10 | 1.5 | 0.1 | < 3 | 0.8 | 0.5 | < 3 | 1.2 | 0.1 | Positive | Positive | Positive |
| Anticomplementary activity, $CH_{50}$/mg | Not established | Not tested | Not tested | < 10 | 0.5 | 0.4 | < 3 | 0.0 | 0.0 | Not established | Negative | Negative |
| Electrophoretic purity, % | ≥ 95.0 | 96.3 | 99.3 | ≥ 95.0 | 98.8 | 99.7 | ≥ 95.0 | 98.6 | 99.6 | Positive | Positive | Positive |
| Fractions composition, number of impurities precipitation bands | ≤ 4 | 4 | 1 | ≤ 1 | 1 | 0 | ≤ 1 | 1 | 0 | Positive | Positive | Positive |
| Antibodies to antistaphylolysin, IU/ml | ≥ 4 | 6,5 | 6,5 | 2 | 4 | 4 | 2 | 4 | 4 | Negative | Negative | Negative |
| Antibodies to versatile virus, IU/ml | ≥ 50 | 50 | 50 | 25 | 25 | 25 | 25 | 25 | 25 | Negative | Negative | Negative |
| Antibodies to HbsAg, IU/g | ≥ 0,5 | 17,8 | 17,7 | ≥ 0,5 | 22,5 | 22,6 | ≥ 0,5 | 20,0 | 19,8 | Negative | Negative | Negative |

Chromatographic purification results in a decline of haemagglutinins amount. If they are present in high amounts in intravenous immunoglobulin medications, haemolytic reactions may occur. This is especially important when administered to premature infants [3, 19]. Anti-A and anti-B haemagglutinins titer must be no more than 1:64. Fifteen tested S/D-treated samples of intravenous immunoglobulins show as low haemagglutinins levels as 1:8–1:16. Preparations are safe in respect of haemolytic reactions.

Bacterial endotoxins cause pyrogenic reactions. According to pharmaceutical regulations, immunoglobulins are considered to be not pyrogenic in the case, if, after being injected to experimental animals, in no one of three temperature readings, a rise (or decline) by more than 0.4 $^0$C is observed. The sum of the maximal body temperature changes must be no more than 1.2 $^0$C.

The testing of 45 full-scale non-treated immunoglobulin batches showed maximum allowable pyrogen values (1.1$\pm$0.3 $^0$C) in 68 % of cases. For S/D treated samples (112 batches) this value amounted to 0.6$\pm$0.3 $^0$C. The difference is statistically significant (1$^{st}$ acceptance probability threshold). Even more, non-treated immunoglobulin samples with pyrogen values in the range of 2.2–4.5 $^0$C (4 cases) showed a temperature decline after S/D treatment to the values as low as 0.4–1.1 $^0$C.

**Conclusions**

The elaborated S/D virus inactivation and chromatographic purification technique ensures the retaining of protein physico-chemical and biological properties. No significant changes in ACA and antibodies activity were observed. The additional removal of trace impurities during chromatographic purification improves quality and safety of pharmaceutical immunoglobulin G preparations.

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# ФІЗИКО-ХІМІЧНІ І БІОЛОГІЧНІ ВЛАСТИВОСТІ ПРЕПАРАТІВ ІМУНОГЛОБУЛІНУ G, ОБРОБЛЕНИХ СОЛЬВЕНТ/ДЕТЕРГЕНТНИМ СПОСОБОМ

Р е з ю м е

Досліджували однорідність білкового складу, фракційний склад, молекулярно-масовий склад, антикомплементарну активність, анти-А та анти-В гемаглютиніни, специфічну активність, пірогени у внутрішньм’язових та внутрішньосудинних препаратах імуноглобуліну G, які проходили та не проходили стадію інактивації вірусів сольвент/детергентним методом. Показано збереження фізико-хімічних та біологічних властивостей препаратів. Завдяки очищенню методами аніоно- та катіонообмінної препаративної хроматографії низького тиску, досягається покращення якості препаратів імуноглобуліну.

К л ю ч о в і с л о в а: препарати імуноглобуліну, інактивація вірусів, сольвент/детергент, хроматографічні методи очищення.

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# ФИЗИКО-ХИМИЧЕСКИЕ И БИОЛОГИЧЕСКИЕ СВОЙСТВА ПРЕПАРАТОВ ИММУНОГЛОБУЛИНА G, ОБРАБОТАННЫХ СОЛЬВЕНТ/ДЕТЕРГЕНТНЫМ СПОСОБОМ

Р е з ю м е

Исследовали однородность белкового состава, фракционный состав, молекулярно-массовый состав, антикомплементарную активность, анти-А и анти-В гемагглютинины, специфическую активность, пирогены во внутримышечных и внутривенных препаратах иммуноглобулина G, проходивших и не проходивших стадию инактивации вирусов сольвент/детергентным методом. Показано сохранение физико-химических и биологических свойств препаратов. Благодаря очистке методами анионо- и катионообменной препаративной хроматографии низкого давления, достигается улучшение качества препаратов иммуноглобулина.

К л ю ч е в ы е с л о в а: препараты иммуноглобулина, инактивация вирусов, сольвент/детергент, хроматографические методы очистки.

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Отримано 22.02.2008