EXPERIMENTAL ARTICLES

UDC 615.37:577.27:543.42 https://doi.org/10.15407/biotech19.03.032

EVALUATION OF A TRIS-GLYCINE BUFFER MODIFICATION FOR ANTICOMPLEMENTARY ACTIVITY TESTING OF IMMUNOGLOBULIN G PREPARATIONS

O. V. YATSENKO[1] *(https://orcid.org/0009-0009-6060-4870)*
M. V. MYLENKO[1] *(https://orcid.org/0000-0002-2662-2137)*
O. M. DYSHLIUK[1] *(https://orcid.org/0000-0002-1841-0716)*
K. O. YEFYMENKO[1] *(https://orcid.org/0009-0001-2347-5389)*
Yu. D. VINNICHUK[2] *(https://orcid.org/0000-0002-3148-8067)*
A. Yu. LABYNTSEV[2] *(https://orcid.org/0000-0002-1793-4630)*
O. S. OSYPCHUK[1] *(https://orcid.org/0009-0000-9224-7577)*

[1] Biopharma Plasma LLC, Bila Tserkva, Kyiv region, Ukraine
[2] Palladin Institute of biochemistry of NAS of Ukraine, Kyiv

E-mail: vinnichukju@gmail.com

Polyvalent human intravenous immunoglobulin (IVIG) is a complex protein preparation obtained from human plasma and requires strict quality control to ensure safety and clinical efficacy. One important quality attribute is anticomplementary activity (ACA), which reflects the potential of immunoglobulin preparations to activate the complement system.

Aim. The study aimed to assess the applicability of the Tris-glycine buffer as an alternative to the pharmacopoeial barbital buffer and to provide practical guidance for laboratories performing ACA testing.

Methods. Two approaches for determining ACA were evaluated: the method described in the European Pharmacopeia (Ph. Eur.) and a modified procedure using a Tris-glycine buffer. Two approaches for determining ACA were evaluated: the method described in the European Pharmacopeia (Ph. Eur.) and a modified procedure using a Tris-glycine buffer. The tested immunoglobulins were obtained by fractionation of donor blood plasma (de-identified blood samples). Complement activity was evaluated by determining the degree of hemolysis of activated complement. ACA was assayed according to the method described in Ph. Eur. 01/2018:20617 by calculating the ratio of the activity of bound complement in the tested sample (solutions of the preparation and standard samples) to its output activity in the control sample. Data were analyzed using linear regression and a two-tailed Student's t-test (Microsoft Excel).

Results. We tested the Tris-glycine buffer-based modification for the ACA assay. The methodology was effectively reproduced and verified to meet the requirements specified in the Ph. Eur. monograph 01/2018:20617. In addition, the workflow has been optimized, increasing analytical productivity by using deep-well plates instead of individual microtubes, reducing hands-on time, and improving reproducibility by minimizing operator-dependent variability. However, when barbital buffer is replaced with a Tris-glycine buffer, samples must be adjusted to pH 7.0 before analysis, as the buffering capacity of Tris-glycine is insufficient to maintain a stable pH in the reaction mixture. Under these conditions, all tested samples met the acceptance criteria, with ACA values below 50% (less than 1 CH_{50}/mL per 1 mg of immunoglobulin), indicating minimal complement activation.

Conclusion. The proposed modification represents a practical optimization of the known method, the ACA assay. It facilitates more efficient and reproducible testing in quality control laboratories, while maintaining compliance with established safety criteria for immunoglobulin G preparations.

Keywords: anticomplementary activity, immunoglobulin preparation, quality control.

Citation: Yatsenko, O. V., Mylenko, M. V., Dyshliuk, O. M., Yefymenko, K. O., Vinnichuk, Yu. D., Labyntsev, A. Yu., Osypchuk, O. S. (2026). Evaluation of a tris-glycine buffer modification for anticomplementary activity testing of immunoglobulin G preparations. *Biotechnologia Acta*, 19(3), 32–45. https://doi.org/10.15407/biotech19.03.032

Polyvalent human intravenous immunoglobulin (IVIG) is a purified and concentrated preparation of the gamma-globulin protein fraction obtained from human blood plasma, which contains high titers of antibodies, primarily immunoglobulin G (IgG). IVIG remains a primary therapy for numerous diseases, being the top immunobiological drug. It is widely used in clinical practice for the prevention and treatment of congenital and acquired immunodeficiencies, as well as certain autoimmune conditions [1–7]. IgG molecules replace missing or inactive native immunoglobulins; therefore, immunoglobulin replacement therapy is considered the "gold standard" for treating primary immunodeficiencies, especially in cases of antibody formation defects [8]. However, the range of diseases treated with non-specific (polivalent) IVIG is continually broadening. The criteria for IVIG administration are based on the U.S. Food and Drug Administration (FDA) guidelines, which state that it can be used for both approved (on-label) and unapproved (off-label) indications. On-label use of IVIG includes: primary/hereditary conditions (such as X-linked agammaglobulinemia, severe combined immunodeficiency, hypogammaglobulinemia) and secondary/acquired immunodeficiency states (for example, pediatric HIV infection), prevention of post-transplant complications (in bone marrow and stem cell transplants), Kawasaki disease, Guillain-Barré syndrome, and other manifestations of chronic demyelinating polyneuropathy; severe myasthenia, anti-D alloimmunization, and others. IVIG is widely used for treating blood system diseases, including idiopathic thrombocytopenic purpura, and is included in treatment protocols for neoplasms of hematopoietic and lymphoid tissues (chronic lymphocytic leukemia, multiple myeloma, Hodgkin's lymphoma, and other lymphoproliferative neoplasms) during the threat or development of infectious-inflammatory complications of bacterial or viral origin, which are caused by decreased IgG synthesis in such pathological conditions. There are reports of successful use of IVIG in treating inhibitor forms of hemophilia A and B, coagulopathy caused by factor XIII inhibitors, and autoimmune hemolytic anemia. IVIG is also widely used in burn medicine [1, 4, 7]. It is a promising immunotherapy approach for the treatment of several diseases affecting the central and peripheral nervous systems, including chronic demyelinating polyneuropathy, multifocal motor neuropathy, and dermatomyositis. Immunoglobulins also serve as an alternative to plasma exchange and standard immunosuppression in cases of decompensation of autoimmune diseases [9–11]. IVIG treatment may be safe and effective for enhancing survival in patients with COVID-19 [12].

The mechanism of action of IVIG is considered one of the most complex among blood products [4]. In primary or secondary immunodeficiency, Ig administration serves as replacement therapy with a clear purpose: restoring IgG levels. However, considering the anti-inflammatory and immunomodulatory properties of Ig, several mechanisms have been proposed to explain its influence on immune system regulation. Specifically: interaction with the specific Fc receptor; regulation of complement pathways and activation of mechanisms that induce solubilization of circulating immune complexes; formation of idiotype-anti-idiotype dimers; modulation of certain cytokines and production of their antagonists; apoptosis of B- and T-cells via activation of the Fas receptor; blocking the interaction between T-cells and superantigens; regulation of self-reactivity and induction of tolerance; suppression of dendritic cell differentiation and maturation. The effect of Ig on regulatory T cells (Tregs), specifically $CD4^+$, $CD25^+$, and $FoxP3^+$, has been described. It has been shown that Ig preparations inhibit the differentiation and expansion of Th17 cells, which, in addition to defending against extracellular pathogens like Klebsiella and Candida, play a vital role in the development of various autoimmune, allergic, and inflammatory diseases [3].

Immunoglobulins are obtained from large pools of human plasma of healthy donors, which provides a diversity of antibodies but also carries the risk of various infections, requiring ongoing efforts to improve safety while maintaining the necessary tolerability of the drug [3, 10, 11, 13]. In recent years, safe preparations with normal half-lives and effector functions have been developed, thanks to modern methods for plasma collection and fractionation. Safety measures that minimize the risks of pathogen transmission, along with core quality control tests, are part of the mandatory batch release procedures [4]. However, despite positive results, immunoglobulin therapy requires an individualized approach for each patient. Therefore, both the effectiveness and safety of intravenous immunoglobulin use are still being

studied, and the results of such studies are actively discussed in contemporary scientific literature [9].

Control measures are implemented before, during, and after IVIG production. Only those Ig products that fully meet the quality control criteria established by law are approved for medical use. Quality requirements are based on standards expected by doctors and patients, as well as the specifications set by registration authorities in different countries comprehensive quality assurance program starts with the careful collection and analysis of each blood donation, continues through all production stages, and concludes with thorough testing of each batch of the final product [3, 14]. Key quality characteristics of IVIG include parameters such as: visual inspection, volume, pH, protein concentration, electrophoretic purity, detection of polymers and aggregates, identity confirmation (reactivity only with human serum), detection of antibodies against surface antigens of hepatitis B virus, potency testing of normal Ig, determination of prekallikrein activity, detection of anti-A and anti-B hemagglutinins, osmolality, and assessment of anti-complement activity [3, 4].

Initial attempts to administer intramuscular immunoglobulins intravenously were highly unsuccessful due to severe side effects resulting from strong complement activation by Ig aggregates [1]. The complement system is a vital defense mechanism of the body, belonging to the non-specific factors of innate immunity, which provides antibacterial and antiviral activity in the blood. This activity is realized after the activation of a cascade of reactions involving enzymes and other protein molecules [15]. It was later clarified that during IVIG production, the Fc fragment of Ig is activated, resulting in the formation of immunoglobulin aggregates that can covalently bind to complement proteins. This activation triggers complement without the need for a specific antigen (spontaneous anti-complement activity), whereas native immunoglobulins bind complement only after specific interactions with antigens. The production of safe preparations was achieved only after the development and implementation of specialized plasma-processing methods in transfusion medicine that prevent the activation of Fc fragments in Ig molecules [1]. Therefore, particular attention is paid to spontaneous anti-complement activity (ACA) and size-dependent composition [16, 17] to ensure the safety of preparations [18–20].

Routine determination of ACA using the modified classical method of Kabat and Meyer (1995) is accepted in the European Pharmacopeia (§2.6.17) as a means to prevent adverse reactions associated with IgG aggregates formed during protein purification and/or viral load treatment. The anti-complement titer analysis is based on the hemolysis of sheep erythrocytes sensitized with antibody, using rabbit complement (10 mg of tested Ig incubated with 20 CH_{50} of rabbit complement). The hemolysis of sheep erythrocytes measures residual complement activity pre-coated with antibodies to erythrocyte membrane proteins. The degree of hemolysis is assessed spectrophotometrically. The graphical interpretation of the results allows for determining the amount of material that binds one unit of complement CH_{50}. Then, ADA is expressed as a percentage of complement consumption relative to the total complement reagent, which is considered 100% [4].

Anaphylactic reactions caused by activation of the complement system are among the severe complications of clinical use of IVIG, and they can manifest directly during or immediately after administration of the drug [1]. A rare side effect is hemolysis, which occurs with high doses of IVIG in patients who do not have blood group O (A (II), B (III), AB (IV)). Anti-A and anti-B hemagglutinins can cause both IgG- and complement-mediated hemolysis [5]. Therefore, establishing a stable testing system for ACA should be a priority during trials and before the drug's batch release [21].

Although the manufacturing processes of IVIG have significantly improved over recent years, the complexity of Ig biological functions and the increasing demand for their use necessitate and justify the search for and scientific improvement of quality control tests for these medicinal products, especially for the treatment of many severe pathological conditions.

Assessment of ACA using traditional methods is based on the monograph of the Ph. Eur. 11.0 (01.2023) 01/2018:20617 [22]. These methods typically require a barbital buffer system and standard titration methods [23–25]. Although these traditional approaches have proven effective, they have limitations regarding productivity and the use of barbiturates. Derivatives of barbituric acid belong to the class of sedative-hypnotic agents (ATC code N05CB02). The use of barbiturate buffer in biochemical reactions requires safety measures, as per the manufacturer's instructions (Sigma-Aldrich), as it can cause skin, eye, and respiratory irritation.

To address these limitations, we evaluated a modified ACA assay in which the barbital buffer is replaced with a Tris-glycine buffer system, which offers several advantages, including non-toxicity, isotonicity, and the ability to provide physiological conditions. Additionally, modifications to deep-well 96-well plates for titration, as well as the use of multichannel pipettes and plate readers to measure optical density, optimize the process by increasing both efficiency and accuracy.

The present study aimed to compare the traditional Ph. Eur. method with the modified approach we developed for determining the ACA of immunoglobulin preparations.

Materials and Methods

Human immunoglobulins were obtained by fractionation of donor blood plasma using a modified P. Kistler and H. Nitschmann method (1962; a modification of the E. Cohn method, 1946). The manufacturing process uses blood plasma from voluntary donors, collected at our Biopharma Plasma network of plasma centers, in accordance with GMP requirements and international safety standards. All samples are completely anonymous and undergo mandatory testing for infectious markers.

To measure the anticomplementary activity of immunoglobulin, a certain amount of the test material (10 mg of immunoglobulin) is incubated with a certain amount of guinea pig complement (20 CH_{50}), and the remaining complement is titrated. ACA is expressed as a percentage of complement consumption relative to the control, set at 100%.

A hemolytic unit of complement activity (CH_{50}) is the amount of complement that, under the given reaction conditions, leads to the lysis of 2.5×10^8 optimally sensitized erythrocytes from the total number of 5×10^8. CH_{50} is a conventional unit, and its value depends on the system's pH, ionic strength, erythrocyte concentration, antibody concentration used for sensitization, reaction time, and temperature [21].

Chemicals

The anticomplementary activity measurement method was modified from Ph. Eur. 01/2018:20617. Instead of gelatine barbital buffer solution, Gelatin-Tris-Glycine Buffer Solution (GTBS) was employed (1 g/L gelatin, 0.15 mM $CaCl_2$, 0.5 mM $MgCl_2$, 140 mM NaCl, 3.3 mM N-tris(hydroxymethyl) methyl glycine (Merck) at pH 7.3). This buffer-substitution modification was due to strict regulations on barbiturate use, especially in Ukraine, and the subsequent difficulties associated with this issue [26–28]. Additionally, a tris-glycine buffer has several benefits: it provides physiological conditions, is less toxic than barbiturate-based buffers, and is commonly used for scientific purposes [29–31]. The chemicals used in this study were purchased from Sigma-Aldrich.

Reference Standard (Human Immunoglobulin for Anticomplementary Activity BRP, EDQM, Y0001994), Guinea Pig Complement (Complement sera from guinea pig, Sigma, S1639), Hemolysin Solution (Anti-Sheep Red Blood Cell Stroma antibody produced in rabbit, Sigma, S1389), sheep red blood cells (RBC) were used as reference materials.

5% suspension of sheep erythrocyte preparation

Erythrocyte (Simesta VAAL LLC, Ukraine) concentration was measured on a spectrophotometer (Second 210 Plus Spectrophotometer, Analytik Jena; 541 nm; distilled water as blank). The optical density of the solution was in the range of 0.61 to 0.63 (the concentration of erythrocytes was about 1×10^9 cells/ml).

Hemolysin and complement titration

Hemolytic serum and complement titrations were performed using a modified method described in Ph. Eur. 01/2018:20617. Deep-well 96-well plates replaced dilutions and titrations in 1.5 ml microtubes with a well volume of 2 ml. Deep-well plate mixing was performed on a plate shaker (PST-60-HL, Biosan) at 1000 rpm for 5 minutes. The deep-well plate with the reaction mixtures was incubated at 37 °C for 60 min in a water bath (Water bath WB-4MS, Biosan) [21].

Sedimentation of unlysed sheep RBCs was performed by centrifugation in deep-well plates at 1000 g for 10 min at 5 °C (Thermo Scientific Multifuge X4 RPro MD).

Sedimentation of unlysed sheep RBCs was performed by centrifugation in deep-well plates at 1000 g for 10 min at 5 °C (Thermo Scientific Multifuge X4 RPro MD).

To measure the optical density, 200 µL samples from the deep well plates were transferred using a multichannel dispenser into transparent polystyrene 96-well plates (Greiner) and read in a microplate reader (Thermo Scientific Multiskan SkyHigh Microplate Reader) at 541 nm. The degree of hemolysis (X) was calculated using equation (1):

$$X = \frac{A_{sample} - A_{0\%}}{A_{100\%} - A_{0\%}} \quad (1)$$

where "A_{sample}" is the optical density of wells with hemolysin dilutions. "$A_{0\%}$" is the average optical density of non-hemolyzed cells, and "$A_{100\%}$" is the average optical density for fully hemolyzed cells.

The results of hemolysin titration are considered reliable if the maximum degree of hemolysis falls within the range of values from 50% to 70%.

Using the graph, the optimal hemolysin dilution was selected (increasing hemolysin concentration did not significantly increase hemolysis). This dilution was defined as one minimal hemolytic unit (1 MHU) in 1 ml. The optimal dilution of hemolysin for the sensitized sheep erythrocytes preparation was 2 MHU/ml. The optimal hemolysin dilution corresponding to 2 MHU in 1 ml is determined based on the graph of the dependence of the degree of hemolysis on the dilution of hemolysin.

Complement solutions of 1:400, 1:500, 1:600 were prepared, and the obtained samples were titrated in 2 parallels, as indicated in the monograph 01/2018:20617 Ph. Eur. The degree of hemolysis was calculated according to equation (1).

A graph was constructed on a logarithmic scale, where $Y/(1\text{-}Y)$ was plotted on the X axis, and the volume of diluted complement (ml) was plotted on the Y axis. A straight line was drawn as close as possible to the points, and the volume of diluted complement was determined at the intersection point with the Y axis (corresponding to 50% hemolysis).

Complement activity was calculated according to equation (2):

$$a = \frac{C_d}{C_a \cdot 5} \quad (2)$$

where "a" is a complement activity in CH_{50}/ml; "C_d" is a complement dilution; "C_a" is the volume of diluted complement resulting in 50% hemolysis (intersection point with the Y axis), and 5 is a scaling factor that considers the number of red blood cells.

Test for anticomplementary activity

Complement activity was evaluated by determining the degree of hemolysis of sensitized sheep erythrocytes. Immunoglobulin concentrate components cause complement binding, residual amounts of which provoke complement-initiated hemolysis of sensitized erythrocytes. The degree of hemolysis linearly depends on the concentration of activated complement.

A guinea pig complement was diluted to 100 CH_{50}/ml in GTBS to A guinea pig complement was diluted to 100 CH_{50}/ml in GTBS to determine the anticomplementary activity.

Depending on the immunoglobulin tested and the study results, the pH was adjusted to 7 with 1 M NaOH (Merck) if necessary.

Tests were performed on the tested immunoglobulins, as well as complement control, and positive and negative ACA controls were prepared using the Human Immunoglobulin for Anticomplementary Activity BRP.

Anticomplementary activity measurement is carried out according to the method described in Ph. Eur. 01/2018:20617 by calculating the ratio of the activity of bound complement in the tested sample (solutions of the preparation and standard samples) to its output activity in the control sample according to equation (3):

$$ACA = \frac{a - b}{a} \times 100 \quad (3)$$

where "a" is the average complement activity in the control sample, and "b" is the activity in the test sample.

Results

Validation of the ACA assessment method requires compliance with several requirements:

- During the titration of hemolysin, the maximum degree of hemolysis was in the range of 50 to 70 %.
- The titration curves of complement for sample preparations, positive and negative controls, as well as the complement control, were straight lines with correlation coefficients $R2 \geq 0.99$ within the range of hemolysis degrees from 15 to 85% and a slope of the lines from 0.15 to 0.40;
- The complement activity in the control complement sample met the requirements, from 80 to 120 CH_{50}/mL.
- The activity of the negative control complement ranged from 10–40%, meeting the requirements of the certificate for the standard sample ACA.
- The activity of the positive control complement was 60–100%, within the requirements of the certificate for the standard sample ACA.

The erythrocytes were sensitized with the hemolytic serum to make them susceptible to

the complement. It was shown that different hemolytic serum dilutions exhibited varying degrees of hemolysis upon reaction with complement. The minimal hemolysis unit required for the complement titration was estimated at 1:200 dilution per minimal hemolytic unit (MHU) and used in subsequent investigations (Fig. 1).

The optimal hemolysin dilution for preparing sensitized sheep erythrocytes is 2 MHO/mL (1:100 dilution).

Applied complement dilutions, among which we show dilutions of 1:600, 1:500, and 1:400 allowed to define the corresponding CH_{50}/mlL points of 174, 175, and 172, respectively (Fig. 2).

Titrations at different dilutions show high linearity in each titration.

The determined complement activity is further used to calculate the degree of complement dilution to obtain an activity of 80–120 CH_{50}/ml in the ACA determination assay.

Several analyses were performed, each of which included a series of complement activity titrations:

- complement control (initial complement solution);
- positive control (PC);
- negative control (NC);
- test solution.ACA calculations were also performed (Table 1).

Complement activity was measured 9 times for the sample and ranged from 93 to 109 CH_{50}/mL, without exceeding the predetermined lower and upper limits of 80 and 120 CH_{50}/mL, respectively. As for the anticomplementary activity of the positive control measured subsequently 9 times, it was in the range of 81 and 87% between the pre-established lower (60%) and upper (100%) limits, respectively. The anticomplementary activity of the negative control was in the range of 14–20%, within the pre-established lower (10%) and upper (40%) limits.

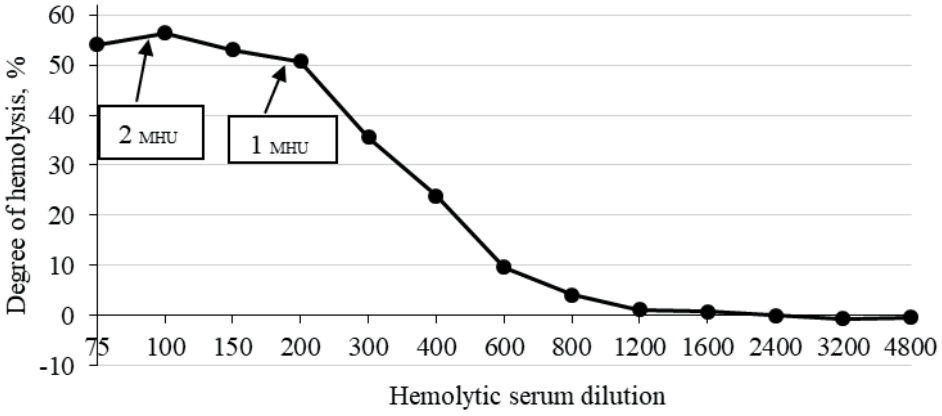


Fig. 1. **Determination of the hemolytic serum titer corresponding to the minimum hemolytic unit (MHU)**

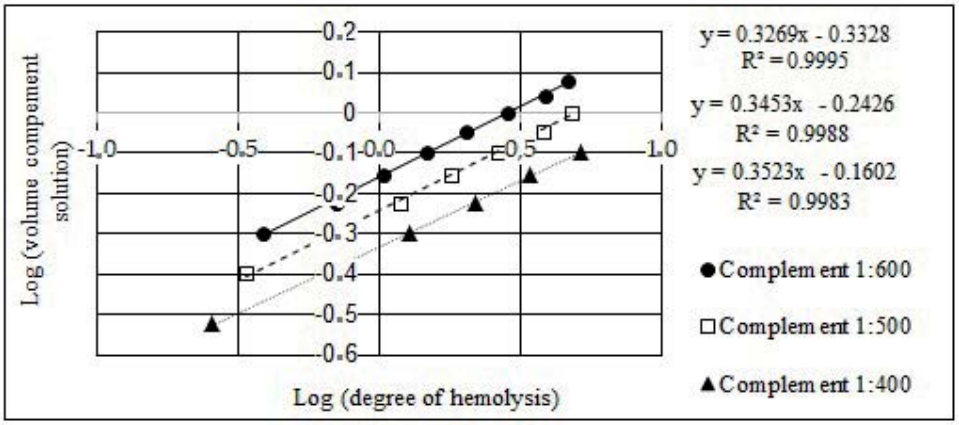


Fig. 2. **Titration of complement at different dilutions**

Table 1. **Preparation of reaction mixtures for the first serological reaction**

Complement activity titrations	GTBS, mL	Immunoglobulin to be examined, mL	Complement solution 100 CH_{50}/mL, mL
Immunoglobulin (50 mg/mL)	0.6	0.2	0.2
Positive control ACA (PC)	–	0.8*	0.2
Negative control ACA (NC)	0.6	0.2*	0.2
Complement control	0.8	–	0.2

* when using Human Immunoglobulin for anticomplementary activity BRP.

The anticomplementary activity of thirty-six different IVIG batches was tested, and it did not exceed the pre-established maximum of 50% (Ph. Eur. 07/2022:0918 'Human normal immunoglobulin for intravenous administration') (Fig. 3).

The discrepancy between the obtained results was less than 5%, confirming the method's good reproducibility.

The slope angle and correlation coefficient data of the "Titration of complement" graphs are presented. The slope and determination

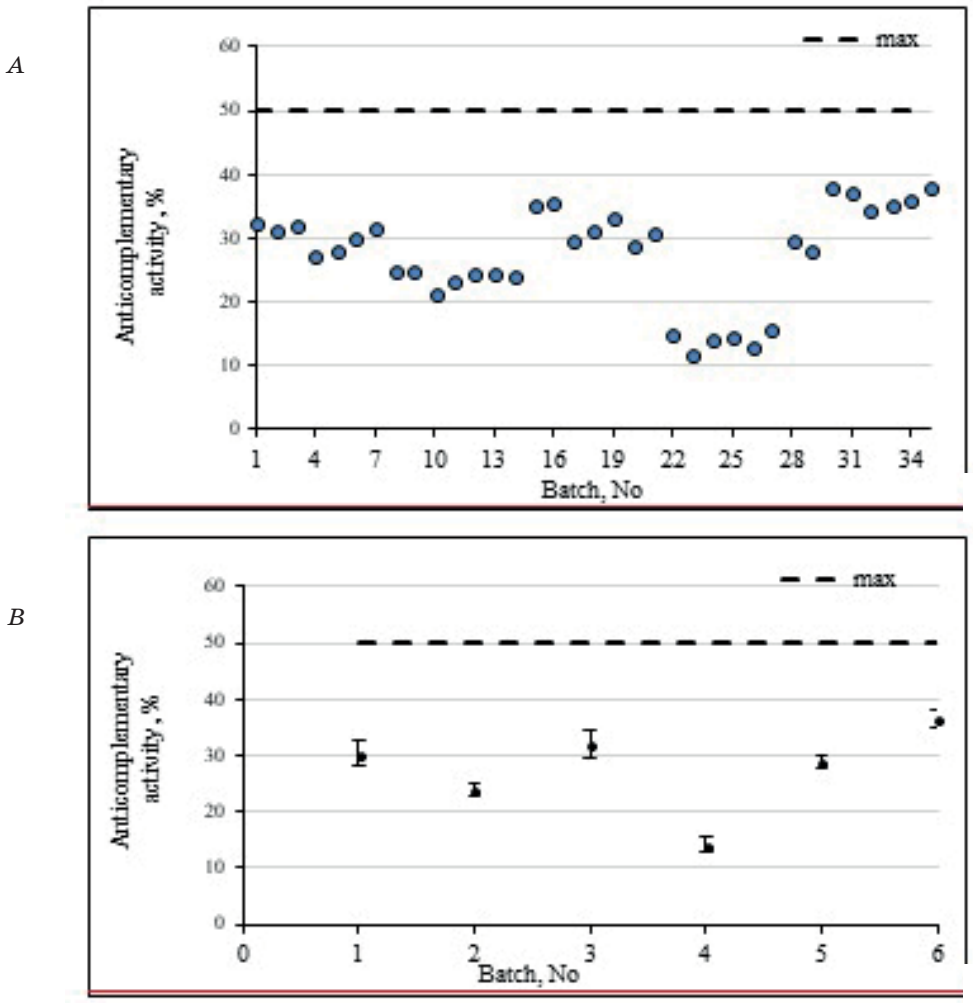


Fig. 3. **Anticomplementary activity of thirty-six different IVIG batches (2022–2024 years)**
The corresponding upper limit is shown: max — upper limit

coefficient R^2 values for the complement titration plots confirmed the assay's reproducibility (Fig. 4).

Interestingly, the European Pharmacopeia [22] used a barbital buffer solution to test anticomplementary activity (Ph. Eur. 01/2018:20617), whereas in our study, we utilized a tris-glycine buffer (Materials and methods). The modification appeared to work perfectly in our hands (Results).

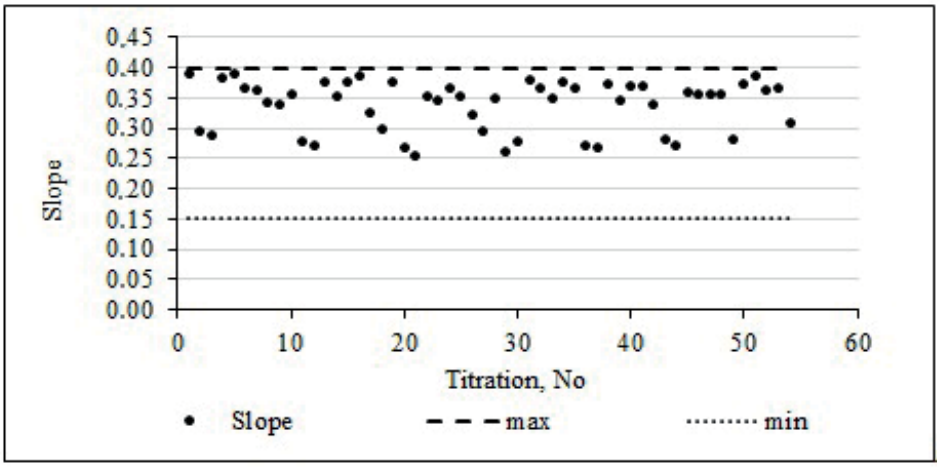
Analyzing deep-well 96-well plates is a more automated approach than analyzing in test tubes. The volumes of reaction mixtures remain unchanged, which does not contradict the monograph's requirements and provides numerous benefits, including time savings, higher accuracy, increased throughput, and a reduced workload.

In general, the method of determining ACA is very complicated since the result is affected not only by the parameters of the ACA test itself: the pH of the samples and the buffer solution; the batch of hemolysin and complement; the BRP control batch; the process of sheep blood collection and erythrocytes sedimentation rate; interpretation of titration results and temporal variations between assay steps.

Also, many things do not allow the analysis to be carried out entirely identically, namely:

- different pipetting techniques, dosing, duration of stages, and sample handling by the personnel;
- as the method is a long-term method, compliance with strict temperature regimes for cooling/heating reaction mixtures, pH of controls and samples;
- The robustness of blood will differ from sheep blood pools and influence the method's accuracy. Moreover, blood can change its properties as a biological material during storage.
- The transparency of the supernatant during erythrocyte suspension washing may also change.

A



B

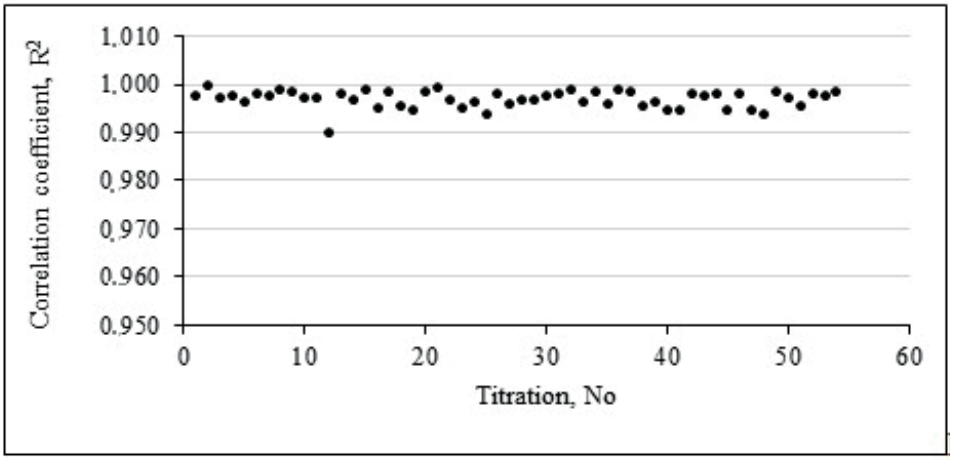


Fig. 4. **Slope (*A*) and determination coefficient R^2 (*B*) values for complement titration plots**
The corresponding upper/lower limits are shown: max — upper limit, min — lower limit

Table 2. **Study of the effect of pH on ACA preparations.**

Series	**Anticomplementary activity, %**		
	Reaction mixtures		
	Without pH adjustment (to 7.0)	With pH adjustment (to 7.0)	Difference, $\|\Delta x_i\|$
1	37.0	30.5	6.5
	31.7		1.2
	34.9		4.4
	37.0		6.5
2	29.6	23.8	5.7
	24.3		0.4
	26.3		2.4
	28.0		4.1
3	36.9	32.0	4.9
	33.1		1.1
	33.6		1.6
	36.7		4.7
4	21.4	14.0	7.4
	19.7		5.7
	20.9		6.9
	20.6		6.6
The sum of the difference values, $\|\Delta x_i\|$			70.1
Average difference value, $\|\Delta \bar{x}\|$			4.38
The standard deviation of difference values, Δ (S_Δ)			2.33
Two-tailed t-test for independent samples: $test\ value = \frac{\|\Delta \bar{x}\|}{S_\Delta} \times \sqrt{n}$			7.510
t, (n – 1, 95%)			2.1314
t, (n – 1, 99%)			2.9467

• Sensitized erythrocyte preparation in larger volumes may require more time to warm the entire volume to a sufficient temperature for effective hemolysin treatment.

We determined the effect of adjusting the pH in immunoglobulin preparations on ACA determination results by calculating two-tailed t-tests for the corresponding samples (Ph. Eur. 11 01/2020:50300).

According to the monograph of Ph. Eur. 01/2018:20617, the pH of immunoglobulin samples must be brought to 7 before incubation with complement.

On the one hand, the pH of the incubation mixture can affect the activity of the complement. On the other hand, adding an agent to increase the pH can cause immunoglobulin denaturation, for example, by forming aggregates near the isoelectric point [32].

The pH of the tested preparations ranges from 4.6 to 5.4. Samples are adjusted to pH 7.0 before preparing the test solutions, and the test solutions have a pH range of 7.0 to 7.2.

Tests on the anticomplementary activity were carried out without adjusting the reaction mixture's pH to 7.0 beforehand. Table 2 shows the results of the preparations' ACA and the two-sided t-test calculations for the corresponding samples.

The Student's t-test was calculated and compared with the tabular value of the Student's distribution to test two independent samples. If the found value is higher than the table value, the difference in the result is considered statistically significant.

Since the test statistic (test value = 7.51) is greater than the tabular t value at the 99% level (t = 2.9467), the difference between the ACA measured with pre-adjusted and unadjusted pH in the reaction mixtures is statistically significant. The measured ACA for reaction mixtures without preliminary pH adjustment is higher than that of samples with preliminary pH adjustment. Therefore, it is necessary to adjust the samples' pH to 7.0 before determining the ACA.

Discussion

Even minor differences in manufacturing processes and formulations can affect clinical effectiveness and tolerability. Given the complexity of the multi-step process for obtaining IVIG from human plasma, strict quality control of the final preparations is necessary [7]. Alternative methods for evaluating ACA therapeutic drugs, such as IVIG, have been proposed by other authors, including radioimmunoassay (RIA) and enzyme-linked immunosorbent assay (ELISA). These methods detect complement activation products, such as C4a (RIA), or the consumption of complement subcomponents, including C1q or C1r (ELISA). The latter method, performed in microtitration plates, is based on forming a complex between aggregated IgG and purified human C1q reagent. According to the authors, the ELISA test is considered more accurate, easier to use, and does not require internal reagents or specialised technical knowledge [4].

Optimised analysis of the qualitative and quantitative determination of C1q-binding aggregates, as well as an immunoassay method for intravenous and intramuscular IgG preparations, was described by other authors [33]. In Japan, the first registered reference material was created to standardise the ACA test, enabling each laboratory to normalise ACA values for IVIG continuously and contribute to the quality control of preparations [20]. According to other studies, a combination of multiple bioanalytical methods can enhance antibody quality control and monitor the IVIG manufacturing process. For example, circular dichroism, surface plasmon resonance, and two-dimensional electrophoresis (2DE) can be used in conjunction [7]. Compared to radioimmunological, ELISA, and other assays, our proposed method is not a complex immunological analysis that requires expensive reagents; the study takes less time and reduces the risk of error.

The complement titration method we developed achieves high accuracy and reproducibility. The workflow has been optimized, increasing analytical productivity by using deep-well plates instead of individual microtubes. The analysis for assessing ACA immunoglobulins was validated by applying the method to deep-well plates with buffer replacement. The methodology was effectively reproduced and verified to meet the requirements specified in the Ph. Eur. monograph 01/2018:20617.

It has been demonstrated that when replacing barbital buffer with an alternative tris-glycine buffer, the samples must be adjusted to pH 7.0 before determining

complementarity activity, because the tris-glycine buffer's buffer capacity is insufficient to maintain the reaction mixture's pH. Since the acceptance criteria were met, the ACA of the preparations measured under these conditions is correct and is less than 50% (less than 1 CH_{50}/mL per 1 mg of immunoglobulin), indicating minimal complement activation.

Optimizing the ACA analysis setup with buffer replacement is a safer and more cost-effective approach. The production of immunobiological drugs in Ukraine complies with the general recommendations established by the International Union of Immunological Societies and the World Health Organisation. Still, they are less accessible for wide-scale use due to the high-cost technology involved in manufacturing and, consequently, the high price of the drug [9]. Therefore, reducing the cost of final product testing remains a relevant issue, as it expands their potential for clinical application.

Performing the methodology on plates enables the simultaneous analysis of multiple samples (20 or more) by reducing the duration of reagent preparation for analysis. It is possible to maintain a uniform temperature condition in each well of a deep-well plate by incubating the plate in a water bath. This results in high linearity and accuracy while minimizing personnel's influence on the reaction's course.

Limitations of the study

The present work is an applied laboratory study focused on practical optimization of the ACA assay. It does not investigate the underlying immunological mechanisms of complement activation, nor does it aim to provide a comprehensive evaluation of all quality control parameters of immunoglobulin preparations.

In addition, the study evaluates a specific buffer modification within the framework of an established pharmacopoeial method. Further validation across a broader range of products and laboratory settings may be required to confirm the general applicability of the proposed approach.

Conclusions

This study demonstrates that the proposed Tris-glycine buffer-based modification represents a practical optimization of the established ACA assay. The modified approach enables more efficient and reproducible testing while maintaining compliance with the applicable acceptance criteria. Implementation of the assay in a microplate format further facilitates the simultaneous analysis of multiple samples, supporting routine application in immunoglobulin G quality control laboratories.

Author Contributions

O. Yatsenko — conceptualization, methodology, validation, formal analysis, investigation, data curation, writing —original draft preparation, writing — review and editing, visualization; M. Mylenko — writing — original draft preparation, writing — review and editing, visualization; O. Dyshliuk — writing— original draft preparation, writing — review and editing, visualization; K. Yefymenko — conceptualization, funding acquisition, resource; Yu. Vinnichuk — writing — review and editing; A. Labyntsev — writing — review and editing; O. Osypchuk — validation, formal analysis, investigation, data curation, writing — review and editing, project administration. All authors contributed to the manuscript's revision and read and approved the submitted version.

Funding

This research was funded by "Biopharma Plasma LLC".

Conflict of Interest

The authors declare no conflict of interests.

Acknowledgments

We thank all of Biopharma Plasma LLC's departments and facilities that supported the study.

Abbreviations

ACA — Anticimplementary activity
IVIG — Inrtavenous immunoglobulin
IgG — Immunoglobulin G
RBC — Sheep red blood cells
FDA — Food and Drug Administration
BRP — Biological Reference Preparation
PC — Positive control
NC — Negative control
RIA — Radioimmunoassay
ELISA — Enzyme-linked immunosorbent assay

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ОЦІНКА МОДИФІКАЦІЇ ТРИС-ГЛІЦИНОВОГО БУФЕРА ДЛЯ ТЕСТУВАННЯ АНТИ-КОМПЛЕМЕНТАРНОЇ АКТИВНОСТІ ПРЕПАРАТІВ ІМУНОГЛОБУЛІНУ G

О. В. Яценко[1] *(https://orcid.org/0009-0009-6060-4870)*
М.В. Миленко[1] *(https://orcid.org/0000-0002-2662-2137)*
О. М. Дишлюк[1] *(https://orcid.org/0000-0002-1841-0716)*
К. О. Єфименко[1] *(https://orcid.org/0009-0001-2347-5389)*
Ю. Д. Вінничук[2] *(https://orcid.org/0000-0002-3148-8067)*
А. Ю. Лабинцев[2] *(https://orcid.org/0000-0002-1793-4630)*
О.С. Осипчук[1] *(https://orcid.org/0009-0000-9224-7577)*

[1] Біофарма Плазма, Біла Церква, Київська область, Україна
[2] Інститут біохімії НАН України, Київ, Україна

E-mail: vinnichukju@gmail.com

Полівалентний внутрішньовенний імуноглобулін людини (IVIG) — складний білковий препарат, отриманий з плазми крові, який вимагає суворого контролю якості, що необхідно для безпечного застосування та клінічної ефективності. Однією з важливих характеристик якості таких препаратів є антикомплементарна активність (АКА), яка відображає потенціал імуноглобулінів активувати систему комплементу.

Мета. Оцінити можливість застосувність трис-гліцинового буфера як альтернативи барбіталовому буферу та надати практичні рекомендації лабораторіям, що проводять тестування АКА.

Методи. Було оцінено два підходи до визначення АКА: метод, описаний у Європейській фармакопеї (Ph. Eur.), та модифікований метод з використанням трис-гліцинового буфера. Імуноглобуліни для досліджень отримували шляхом фракціонування донорської крові (повністю анонімні зразки). Активність комплементу оцінювали, визначаючи ступінь гемолізу сенсибілізованих еритроцитів барана, який лінійно залежить від концентрації активованого комплементу. АСА визначали за методом, описаним у Ph. Eur. 01/2018:20617, шляхом розрахунку співвідношення активності зв'язаного комплементу в досліджуваному зразку (розчини препарату та стандартних зразків) до його вихідної активності в контрольному зразку. Дані аналізували за допомогою лінійної регресії та двостороннього t-критерію Стьюдента (Microsoft Excel).

Результати. Модифікація на основі трис-гліцинового буфера для аналізу АКА (антикомплементарної активності) показала, що методологію було ефективно відтворено та перевірено відповідно вимогам, зазначеним у монографії Ph. Eur. 01/2018:20617. Крім того, робочий процес було оптимізовано, що підвищує аналітичну продуктивність методу завдяки використанню глибоколункових мікропланшетів, що, в свою чергу, скорочує час аналізу та покращує відтворюваність, мінімізуючизалежну від людського фактору варіабельність. Однак, при заміні барбіталового буферу на трис-гліциновий, перед аналізом необхідно довести pH зразків до 7,0, оскільки буферна здатність трис-гліцину недостатня для підтримки стабільного pH у реакційній суміші. За таких умов усі протестовані зразки відповідають критеріям прийнятності, зі значеннями АСА нижче 50% (менше 1 CH50/мл на 1 мг імуноглобуліну), що свідчить про мінімальну активацію комплементу.

Висновки. Запропонована модифікація являє собою практичну оптимізацію відомого методу визначення АКА, яка забезпечує більш ефективне та відтворюване тестування у лабораторіях контролю якості, зберігаючи при цьому відповідність встановленим критеріям безпеки препаратів імуноглобуліну G.

Ключові слова: антикомплементарна активність, препарати іммуноглобуліну, контроль якості.

Received 2026/03/27
Revised 2026/05/20
Accepted