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PREPARATION OF EFFECTIVE DIAGNOSTIC REAGENTS SUITABLE FOR ELISA IN THE DIAGNOSIS OF *BACILLUS ANTHRACIS*

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ABSTRACT

The aim of this study was to optimize methods for precipitating gamma-globulins from hyperimmune sera obtained from animals immunized with *Bacillus anthracis* strains, followed by the preparation of immunoperoxidase conjugates for use in enzyme-linked immunosorbent assays (ELISA).

Two methods for gamma-globulin isolation were compared: ammonium sulfate precipitation and multi-step ethanol fractionation according to a modified Kon method. Protein yield, purity, and precipitating activity in the radial diffusion in gel (RDG) assay were evaluated. The results showed that the ethanol method provided higher gamma-globulin yield (up to 85 %) and protein concentration (17.4 mg/mL) compared to ammonium sulfate precipitation. Maximum precipitating activity (1:16) was achieved using the optimized ethanol precipitation, whereas the activity obtained with ammonium sulfate was 1:8.

The isolated fractions were conjugated with horseradish peroxidase using the periodate oxidation method. Spectrophotometric analysis at 280 nm and 403 nm demonstrated that conjugates prepared from ethanol-precipitated gamma-globulins exhibited more uniform enzymatic activity and stable optical properties.

These results indicate that ethanol fractionation is an effective approach for obtaining immunoperoxidase conjugates suitable for ELISA-based detection of *Bacillus anthracis*.

Keywords: *Bacillus anthracis*, immunoperoxidase conjugate, gamma globulin, ammonium sulfate, Cohn alcohol fractionation method.

INTRODUCTION

Bacillus anthracis is an acute zoonotic infectious disease caused by a spore-forming bacterium with high resistance to adverse environmental conditions. The pathogen belongs to the family *Bacillaceae* and is a non-motile, aerobic, Gram-positive rod measuring 6–10 µm in length and 1–2 µm in width, capable of forming spores and a polysaccharide capsule [1].

The disease is registered in sporadic cases among both farm animals and humans in many countries around the world [2, 3]. In Kazakhstan, *Bacillus anthracis* is a pressing epizootic problem: there are natural soil and epizootic foci throughout the country, especially in areas where animals infected with *B. anthracis* were previously buried [2, 4, 5].

The relevance of this issue increased dramatically after the bioterrorist attacks in the United States in the fall of 2001, when *B. anthracis* spores were used as a biological weapon. According to the CDC, 22 cases of the disease were recorded at that time, including 11 cutaneous and 11 inhalation forms, of which 5 were fatal despite timely antibiotic therapy [6].

Given the pathogen's resilience, epidemiological significance, and potential danger as a biological agent, issues related to the specific and sensitive detection of *B. anthracis* are of particular importance. Currently, active work is underway to improve existing diagnostic methods, including ELISA, as well as to develop new approaches to the detection of *B. anthracis* spores and antigens [7].

In the laboratory diagnosis of *Bacillus anthracis*, the detection and identification of *Bacillus anthracis* in biological samples and the environment plays a key role [8, 9]. One method

for diagnosing *Bacillus anthracis* is ELISA. ELISA is widely used to detect *B. anthracis* antibodies and antigens, including in diagnostic kits for screening animals and humans [10, 11]. One of the key elements of ELISA is an immune conjugate – an antibody linked to an enzyme (e.g., horseradish peroxidase) that provides visualization of the reaction. The effectiveness of the conjugate directly depends on the quality of the source immunoglobulins and the conditions under which they are obtained.

Gamma globulins isolated from the serum of immunized animals are often used as a basis for creating conjugates. The precipitation stage plays a crucial role in preparing the sample for further purification and conjugation. However, failure to comply with the conditions may result in protein denaturation, loss of specificity, and a decrease in the yield of the target fraction [12].

Optimization of gamma globulin precipitation conditions (e.g., selection of ethyl alcohol concentration, ammonium sulfate concentration, temperature, pH, incubation time) not only increases yield but also preserves antibody functional activity, which is critically important in the production of diagnostic reagents [13, 14].

The aim of this study is to optimize the precipitation of gamma globulins from hyperimmune serum to obtain active conjugates used in ELISA for the detection of *Bacillus anthracis* antigen.

MATERIALS AND METHODS

Two methods were used to isolate gamma globulins: precipitation with ammonium sulfate and fractionation using alcohol.

Ammonium sulfate precipitation

Ammonium sulfate was pre-dried at 37°C for 1–2 days. To prepare a 50% saturated solution, 367.2 g of the dried reagent was dissolved in 1 L of sterile distilled water, the solution was heated to 90°C and filtered hot through a double paper filter. After cooling, the solution was acidified to pH 5.5–5.7 using 0.1 N H₂SO₄.

The initial serum was diluted in an equal volume of 0.15 M NaCl, after which an equal volume of the prepared ammonium sulfate solution was added. The mixture was kept at 4 °C for 60 minutes and centrifuged at 3000 rpm for 30 minutes. The gamma globulin precipitate was dissolved in 0.15 M NaCl (1/10 of the volume of the initial serum) and dialyzed against 0.15 M NaCl at 4 °C with dialysate replacement 3 times a day until the reaction with 10% BaCl₂ disappeared. After dialysis, the protein was filtered through a Zeitz CF filter.

Alcohol fractionation

The serum was subjected to multi-stage precipitation with chilled ethanol.

Stage I: at a temperature of minus 5°C, 53% ethanol (883 ml per 1 liter of serum) was added to the serum, which had been adjusted to a pH of 7.0–7.3. The mixture was incubated for 1–2 hours, then centrifuged (12,000 rpm, minus 5 °C).

Stage II: The resulting precipitate was dissolved in physiological saline (1:1.5 by weight), acidified to pH 5.0–5.1, and cooled 26% ethanol (1:1) was added. The mixture was incubated at minus 5°C overnight, then centrifuged.

Stage III: The supernatant containing gamma globulin was alkalized to pH 7.0, the ionic strength was adjusted to 0.05 by adding NaCl, and the protein was precipitated by adding 157 ml of 53% ethanol per 1 liter. The mixture was kept at minus 5°C and centrifuged. The resulting precipitate was dissolved in physiological saline and filtered.

The concentration of total protein in the dissolved precipitate was determined spectrophotometrically at a wavelength of 280 nm, using a calibration curve based on a standard bovine serum albumin (BSA) solution. Protein yield (%) was calculated as the ratio of protein mass in the dissolved precipitate (concentration x volume) to protein mass in the original serum, multiplied by 100 [15, 16].

To assess the purity of the fraction, the composition of the protein fractions (albumins, beta- and gamma-globulins) was analyzed using horizontal electrophoresis in agarose gel. The degree of contamination was determined as the total proportion of albumins and beta-globulins in the total protein, expressed as a percentage [17, 18].

SDS-PAGE analysis of gamma-globulin preparations

The quality of the obtained gamma-globulin preparations was evaluated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing and non-reducing conditions. Prior to electrophoresis, the samples were mixed with an SDS-containing sample buffer. For analysis under reducing conditions, a reducing agent was added to the sample buffer, whereas samples analyzed under non-reducing conditions were prepared without a reducing agent. Electrophoresis was performed using a polyacrylamide gel consisting of stacking and resolving gels. A Novex Sharp Pre-

Stained Protein Standard (Invitrogen, Waltham, MA, USA) was used as a molecular weight marker for electrophoretic calibration. Following electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250 (CBB R-250), and the resulting protein profiles were evaluated based on the distribution and relative intensity of the protein bands.

Conjugation of gamma globulins with horseradish peroxidase

Conjugation of gamma globulin with horseradish peroxidase (HRP, Sigma-Aldrich, ≥250 U/mg, RZ ≥ 3) was performed using the periodate oxidation method (Nakane & Kawaoi, mod. Wilson & Nakane).

HRP (16 mg) was dissolved in 4 ml of H₂O, 0.8 ml of 0.1 M NaIO₄ was added, and the mixture was incubated for 20 minutes. The reaction was stopped by adding ethylene glycol, then dialyzed against 0.01 M carbonate buffer (pH 9.5). IgG (0.5 ml) was diluted with 2.5 ml H₂O and dialyzed with the same buffer for 18 hours. The conjugation reaction was carried out with stirring for 3 hours, NaBH₄ (0.32 ml) was added, and incubated for another 2 hours at 4 °C in the dark.

The conjugate was purified by gel filtration on a column with Sepharose G-200 in phosphate-buffered saline (pH 7.4). The eluates were monitored spectrophotometrically and tested in ELISA [19, 20].

Controls in ELISA

To assess the specificity of the obtained immunoperoxidase conjugates and to control the validity of the ELISA results, positive and negative control samples were included in each assay. The positive control was represented by *Bacillus anthracis* antigen and was used to confirm the ability of the conjugates to specifically recognize the target antigen and to ensure the functionality of the immunoenzymatic detection system. Negative controls included antigens of other infectious agents, namely *Bacillus cereus*, *Pasteurella* spp., *Clostridium* spp., and *Brucella* spp. These samples were used to assess the specificity of the conjugates and to determine the absence of nonspecific reactions with heterologous antigens.

The control samples were analyzed under the same experimental conditions as the test samples, including the same conjugate and substrate reaction conditions. The positive control was expected to produce a specific color reaction, whereas the negative control samples were used to verify the absence of significant nonspecific staining. The results obtained for the control samples were taken into account when interpreting the reactions of the test samples. A valid assay required a clearly detectable reaction with the positive control and no specific reaction with the negative control samples.

The inclusion of positive and negative controls made it possible to distinguish specific antigen–antibody interactions from nonspecific reactions and to evaluate the specificity and reliability of the immunoperoxidase conjugates obtained using gamma-globulins isolated by ammonium sulfate precipitation and alcohol fractionation.

Statistical analysis.

All experiments were performed independently in triplicate (n=3). Results are presented as mean ± standard deviation (SD). Differences between the ammonium sulfate precipitation and alcohol fractionation methods were analyzed using Student's t-test. Differences were considered statistically sig-

nificant at $p < 0.05$.

RESULTS AND DISCUSSION

The aim of the work was to optimize methods for precipitating gamma globulins from hyperimmune sera obtained from animals immunized with *Bacillus anthracis* strains, with subsequent use of the isolated proteins to obtain immune conjugates.

To achieve this goal, two precipitation methods were tested: ammonium sulfate and multi-stage alcohol fractionation.

Ammonium sulfate precipitation

The addition of a 50% ammonium sulfate solution to the diluted serum resulted in the precipitation of proteins, mainly gamma globulins. The average protein yield was about 75% of the total protein content in the original serum, and the concentration of total protein in the dissolved precipitate reached 10.2 mg/ml. However, electrophoretic analysis showed the presence of residual amounts of albumin and beta-globulins, indicating the need for an additional purification step. The degree of contamination was estimated at 15%.

Alcohol fractionation

At the initial stage of the study, a multi-stage protein precipitation procedure using chilled ethanol (alcohol precipitation No. 1) was used. This method provided a higher degree of gamma globulin purification compared to ammonium sulfate precipitation. The average yield of the target protein was about 85%, with a concentration of up to 17.4 mg/ml. The advantage of the alcohol method was a more effective separation of protein fractions, especially in the final stages of precipitation.

However, the second stage, aimed at separating beta and gamma globulins, proved to be sensitive to environmental conditions: part of the gamma fraction remained in the sediment, which required re-purification. Even slight deviations in pH had a significant effect on the efficiency of separation. Taking into account the previously noted limitations, a second variant of alcohol precipitation (method No. 2) was used, with strict adherence to the temperature regime, pH, and 18-hour incubation. This variant made it possible to obtain gamma globulins with a high degree of purity immediately, without the need for repeated precipitation. This significantly increased reproducibility and simplified the process of obtaining the fraction. The summarized comparative indicators of the two approaches to gamma globulin precipitation are presented in Table 1.

SDS-PAGE analysis of gamma-globulin preparations

The gamma-globulin preparations obtained by ammonium sulfate precipitation and multistage alcohol fractionation demonstrated differences in protein composition and purity, as determined by SDS-PAGE under reducing and non-reducing conditions (Figure 1A,B). Under reducing conditions, the

preparation obtained by multistage alcohol fractionation (Figure 1A, lane 1) and the preparation obtained by ammonium sulfate precipitation (Figure 1A, lane 2) showed prominent protein bands at approximately 50 and 25 kDa, corresponding to the heavy and light chains of immunoglobulin G, respectively. The ammonium sulfate preparation additionally contained bands associated with residual serum proteins, including albumin and beta-globulins, whereas these additional protein bands were not detected in the alcohol-fractionated preparation. Under non-reducing conditions (Figure 2B), both preparations showed a predominant band corresponding to intact immunoglobulin G. The SDS-PAGE results confirmed the higher purity of the gamma-globulin preparation obtained by multistage alcohol fractionation.

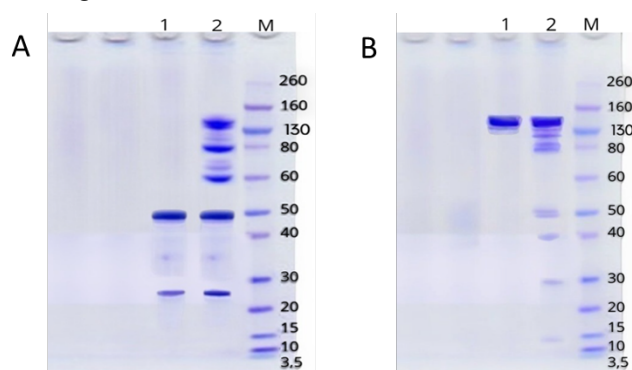
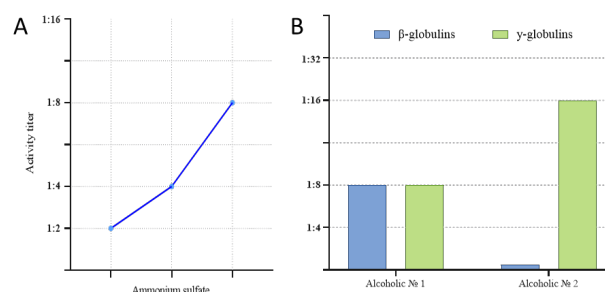


Figure 1. SDS-PAGE analysis of gamma-globulin preparations obtained by different fractionation methods. (A) Reducing conditions; (B) non-reducing conditions. lane 1 – multistage alcohol fractionation; lane 2 – ammonium sulfate precipitation; M – molecular weight marker.

To evaluate the immunological activity of the fractions, DPR was used in agar gel with the *Bacillus anthracis* pathogen antigen. The results are presented in diagram 1.



A – Titre of globulin isolated with ammonium sulphate. **B** – Titre of globulins isolated with alcohol. Gamma-globulins obtained by ammonium sulfate precipitation retained precipitating activity up to a 1:8 dilution.

In the primary ethanol precipitation (method №1), the beta-globulin fraction exhibited activity – positive reactions were observed up to a 1:8 dilution. The gamma-globulin fraction showed a similar result up to 1:8, slightly lower than the

Table 1. Comparative characteristics of gamma globulin precipitation methods

№	Method	Advantage	Restrictions
1	Ammonium sulfate	Simplicity, good protein output	Low selectivity, requires purification
2	Alcoholic	Higher purity and yield, suitable for ELISA	Requires strict adherence to conditions, repeat stage possible

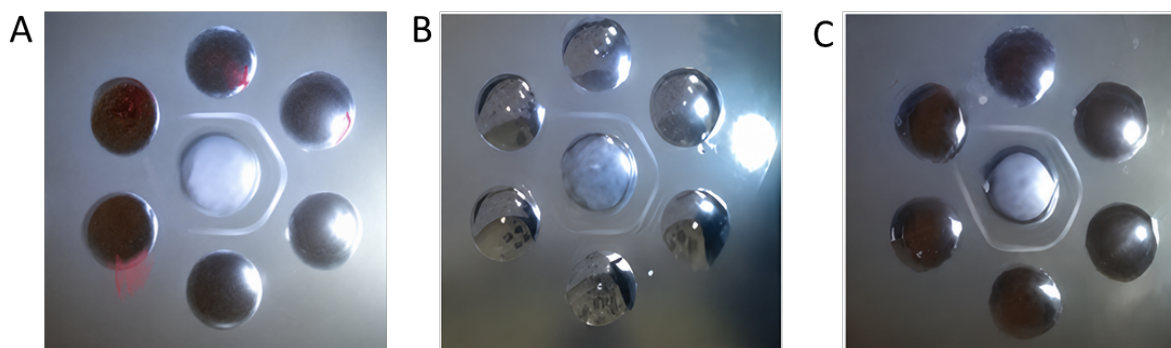


Figure 2. Activity of isolated gamma globulins in DPP. Visualization of the diffusion precipitation reaction (DPR) in agar gel with *Bacillus anthracis* antigen, performed to evaluate the activity of gamma globulins obtained by various methods: A – ammonium sulfate; B – alcohol precipitation No. 1 (first precipitation); C – alcohol precipitation No. 2 (repeat precipitation)

activity observed with ammonium sulfate. The presence of precipitation in the beta fraction is likely due to incomplete separation and partial co-precipitation of gamma-globulins during the first stage.

Repeated alcohol precipitation (the same method No. 1, second stage) allowed the residual activity in the beta fraction to be eliminated. At the same time, gamma globulins showed stable precipitating activity across the entire dilution range, up to 1:16. This indicates a more complete isolation of the target fraction and an increase in the concentration of specific immunoglobulins.

During 18-hour incubation in the alcohol precipitation process (alcohol precipitation No. 2), beta-globulin activity was also not recorded. Gamma globulins, on the contrary, provided a stable reaction at all dilutions, including 1:16, without

the need for an additional precipitation step. This may indicate a more effective and specific separation of fractions already at the first stage of the procedure.

For further production of immunoperoxidase conjugates, two fractions of gamma globulins isolated by different methods were selected. Conjugate No. 1 was obtained from gamma globulins precipitated with ammonium sulfate, and conjugate No. 2 was obtained from gamma globulins isolated by alcohol method No. 2.

To evaluate the quality of immunoperoxidase conjugates obtained from gamma globulins isolated by different methods, spectrophotometry was performed at wavelengths of 280 nm (total protein concentration) and 403 nm (presence of active peroxidase). The results are presented in Table 2 and Figure 3.

The highest optical density values at 403 nm, corresponding to peroxidase enzymatic activity, were recorded in fractions 7 and 8 of conjugate No. 1 (OD403 = 0.631 and 0.650, respectively), as well as in fractions 7, 8, and 9 of conjugate No. 2 (OD403 = 0.602, 0.681, and 0.435). This indicates the presence of an active label and the effectiveness of the conjugation reaction.

Additionally, it should be noted that conjugate No. 2, obtained on the basis of gamma globulins isolated by the alcohol method, demonstrated a more uniform distribution of enzymatic activity between fractions and less pronounced peaks. This may indicate a more stable conjugate structure and effective binding of the enzyme to the protein.

When measured at a wavelength of 280 nm, conjugate No. 2 also showed higher optical density values in most fractions, indicating an increased protein concentration. Taken together, these data confirm the advantage of the alcohol precipitation method for gamma globulins in obtaining stable and functionally active immune conjugates suitable for use in ELISA.

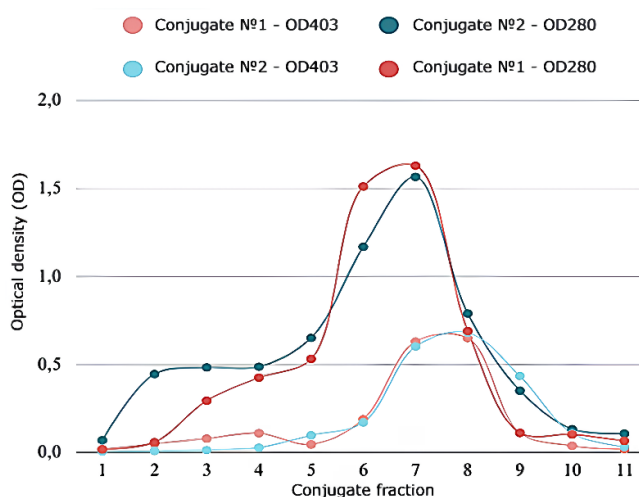


Figure 2. Optical density values of conjugates at wavelengths of 280 and 403 nm.

Table 2. Optical density values of conjugates at wavelengths of 280 and 403 nm.

Serial№	Immunoperoxidase conjugates	Wave-length, nm	Optical density values										
			Conjugate fraction numbers										
			1	2	3	4	5	6	7	8	9	10	11
1	Conjugate №1	280	0,018	0,059	0,295	0,426	0,532	1,512	1,630	0,691	0,113	0,103	0,067
		403	0,02	0,05	0,08	0,11	0,047	0,189	0,631	0,650	0,112	0,038	0,018
2	Conjugate №2	280	0,07	0,446	0,484	0,488	0,652	1,169	1,567	0,790	0,352	0,133	0,108
		403	0,006	0,009	0,014	0,028	0,098	0,171	0,602	0,681	0,435	0,108	0,033

To assess the uniformity of protein distribution and enzymatic activity within the conjugates, coefficients of variation (CV) were calculated for OD280 and OD403 according to the classical formula ($CV = \sigma/\mu \times 100$). Conjugate No. 2 showed lower CV values for both indicators, indicating its better stability and uniformity.

The results obtained demonstrate that the gamma globulin precipitation method has a key influence on the purity and functional activity of the proteins obtained, as well as on the efficiency of their further conjugation with peroxidase. The ammonium sulfate precipitation method is widely used for the preliminary isolation of immunoglobulins from serum due to its simplicity and accessibility [14].

Ammonium sulfate precipitation is a widely used and accessible method for the initial fractionation of immunoglobulins from serum. Fishman and Berg (2018) described ammonium sulfate fractionation as a conventional approach for IgG isolation; however, the method does not provide complete purification, since other serum proteins may remain associated with the precipitated immunoglobulin fraction. Therefore, ammonium sulfate precipitation is generally considered an initial rather than a final purification step when highly purified immunoglobulins are required for the preparation of enzyme-based diagnostic reagents [21]. In the present study, despite a satisfactory protein yield of approximately 75%, ammonium sulfate precipitation resulted in the presence of residual albumin and other serum protein fractions, as demonstrated by SDS-PAGE analysis, indicating the need for additional purification.

Affinity chromatography based on Protein A or Protein A/G represents an alternative approach for the selective purification of immunoglobulins. Salimi et al. (2018) investigated magnetic SiO₂ microspheres coupled with Protein A or Protein A/G for IgG purification and demonstrated efficient isolation of IgG from rabbit serum, with a purity exceeding 95% under optimized conditions [22]. Such affinity-based approaches provide a high degree of selectivity for immunoglobulin purification; however, their implementation requires specialized affinity sorbents and chromatographic or magnetic separation equipment, which may increase the technical complexity and cost of diagnostic reagent production. In the present study, Protein A/G affinity purification was not experimentally evaluated; therefore, no direct experimental comparison with this approach was performed.

In contrast, the multistage alcohol fractionation method used in the present study provided a higher yield and protein concentration than ammonium sulfate precipitation, while SDS-PAGE analysis demonstrated a lower level of contaminating serum proteins. These findings indicate that multistage alcohol fractionation may provide a relatively simple and reproducible alternative for obtaining gamma-globulin fractions suitable for subsequent preparation of immunoperoxidase conjugates. Thus, although affinity chromatography may provide a higher degree of purification, multistage alcohol fractionation may be advantageous when a balance between purification efficiency, reproducibility, technical simplicity, and production cost is required.

CONCLUSION

The results of the study showed that the choice of gamma-

globulin precipitation method significantly affects protein yield, purity, and subsequent biological activity. A direct comparison of ammonium sulfate precipitation and multistage alcohol fractionation demonstrated the advantages of the latter as a more effective method for obtaining gamma-globulin preparations. The alcohol fractionation method provided a higher protein yield and concentration, as well as a higher degree of purity, as confirmed by electrophoretic analysis. The higher immunological activity of the alcohol-fractionated preparation was confirmed by the results of the diffuse precipitation reaction.

Immunoperoxidase conjugates obtained from the alcohol-fractionated gamma-globulin preparations demonstrated higher enzymatic activity and a more uniform distribution of protein and enzyme activity across the chromatographic fractions, indicating effective conjugation and stable properties of the resulting preparations. These findings support the use of multistage alcohol fractionation for the preparation of gamma-globulins intended for the development of diagnostic reagents, particularly immunoperoxidase conjugates for ELISA-based detection of specific antibodies to *Bacillus anthracis*.

Thus, multistage alcohol fractionation represents a promising approach for obtaining gamma-globulin preparations with a favorable combination of yield, purity, and biological activity. The method may be considered suitable for the preparation of immune conjugates for use in ELISA-based diagnostic systems.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript. B.K.U., O.N.S., N.K.O., A.A.I. – conceptualization, data curation, investigation, visualization, writing – original draft, M.Z.K., A.K.A., M.S.S. – methodology and formal analysis, R.Z.N., Z.K.K. – review & editing.

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