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Comparative evaluation of purification strategies for anti-rabies immunoglobulins: Impact on yield, functional activity, and stability

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Abstract

The study aimed to evaluate the effectiveness of different purification methods for anti-rabies immunoglobulins obtained from the plasma of hyperimmunised cattle. The study included a total of 30 animals, randomly divided into three groups based on the purification method: ammonium sulphate precipitation, affinity chromatography on a Protein G column, and a combined method with the addition of ultrafiltration. The purification methods differed in their ability to preserve protein yield, specific antibody activity, and virus-neutralising capacity. The combined protocol produced the highest final protein concentration; however, total protein was interpreted only as an indicator of recovery and concentration efficiency, not as evidence of IgG purity. The superiority of the combined method was supported by the parallel increase in anti-rabies IgG reactivity and virus-neutralising activity. In contrast, the method of precipitation with ammonium sulphate revealed the lowest values for each of these parameters, indicating the lower selectivity of this method and the loss of the functionally active immunoglobulins. However, the results obtained with the precipitation method, Protein G affinity chromatography and ultrafiltration suggest that these methods increase both the amount of anti-rabies immunoglobulin that is obtained and the quality of that immunoglobulin. The method of precipitation with ammonium sulphate and the storage of the obtained immunoglobulin without the step of freezing revealed the greatest decrease in each of the indicators over the 60-day period. However, the method that employed the use of all three steps revealed the best results in the preservation of the protein, specific antibodies, and virus-neutralising activity, with only a 4% loss of each of these initial values after storage at -20°C. Statistical analysis using repeated measures ANOVA confirmed the significant influence of the purification method and storage conditions on all the parameters studied ($p < 0.05$). In conclusion, the integration of precipitation, affinity chromatography, and ultrafiltration resulted in a better yield, functional activity, and stability of anti-rabies immunoglobulins. The practical significance of the present findings lies in the possibility of applying them in the development, standardisation, and production of highly effective immunoglobulin preparations for the prevention and treatment of rabies in veterinary and medical practice.

1. Introduction

Rabies remains one of the most dangerous zoonotic infections, characterised by an almost 100% fatality rate after the onset of clinical symptoms in both animals and humans (Ginayatov et al. 2025; Nurmukhambetova et al. 2025). Despite the availability of effective prevention methods, including vaccination and passive immunisation, the development of creating highly effective and stable immunoglobulin preparations remains challenging in the context of growing epidemiological threats (Fontana et al. 2020). This problem is particularly acute in several Central Asian countries, including the Republic of Kazakhstan, where rabies remains an endemic disease and access to modern medical and veterinary immunobiological preparations is often limited, especially in rural and remote regions. In such conditions, it is necessary to optimise the technological processes for the production of specific immunoglobulins to ensure high efficacy, safety, and accessibility. Besides obtaining a high yield of antibodies, it is equally important to ensure that the antibodies retain their biological activity throughout the production process (Woznichak et al. 2021). Methods like ammonium sulphate precipitation are often the first to be used in the production of immunoglobulins due to their simplicity and low cost. However, the use of these methods is often associated with the loss of both the total and immunoglobulin fractions of the proteins, as well as the decrease of the biological activity of those immunoglobulins. Thus, an analysis of the methods for producing rabies immunoglobulins is appropriate and relevant. Current challenges in rabies prevention require an improvement of the technologies used to produce immunoglobulins, especially in countries like Kazakhstan (Ginayatov et al. 2025). The study did not consider the possibility of combining precipitation technologies with other immunoglobulin production technologies. Therefore, an important question is whether combining different purification methods can lead to an increase in the efficiency of the production process.

Another of the challenges to be faced in the production of immunoglobulins is the selectivity of the purification processes for the IgG fraction of the immunoglobulins - the fraction responsible for initiating the specific immune response to the rabies virus. Research into the use of affinity chromatography to separate the IgG fraction from the other immunoglobulin fractions has revealed that while the process can lead to the production of a purer immunoglobulin preparation, the process can lead to losses in the amount of protein that

is present in the immunoglobulins. Nurmukhambetova et al. (2025) used affinity chromatography to separate the IgG fraction of the immunoglobulins using Protein G columns, leading to their selective incorporation into the columns. As a result, the researchers found that while the process was highly selective in its use of the columns, the yield of the immunoglobulins was decreased. Furthermore, the stability of the immunoglobulins produced at different temperatures was not assessed in this study. The maintenance of the stability of immunoglobulins during storage is another separate issue that is crucial for the production of products with a long shelf life. Even at low temperatures, protein degradation and a decrease in the functional activity of the antibodies can occur. Bitanova et al. (2024) performed studies on the freezing of immunoglobulins at -20 °C. As a result of long-term storage of the immunoglobulins, changes in the structure of the protein molecules were detected. However, the experiment did not include a comparison of the destruction of the protein according to the methods of immunoglobulin purification. The functional activity of IgG can be measured with ELISA and virus neutralisation methods. The activity of IgG depends on the technology used to purify the immunoglobulins. Fontana et al. (2020) studied the effect of various fractionation conditions on the activity of specific IgG. It was concluded that the preservation of the active sites of the antibodies depends on the strength of the ionic interactions between the antibodies and protein components during the purification of the immunoglobulins. However, the authors did not investigate the effect of combined purification methods.

Another problem is the insufficient reproducibility of results when scaling up purification processes from the laboratory to the industrial level. In this context, it is necessary to consider both the yield of the final product and its homogeneity and biological activity. Minor changes in the process parameters of traditional purification methods have significantly affected composition of the final preparation (Woznichak et al. 2021). However, this study did not evaluate the combined purification methods, limiting the applicability of its conclusions in industrial conditions. Furthermore, the use of non-affinity methods leads to the contamination of the final product with non-target proteins and other impurities. In addition to purification challenges, reliable assessment of the specific immune response is also necessary. In this regard, an ELISA was developed and validated that allows for the simultaneous detection of IgM and IgG against the rabies virus (Zajac et al. 2023). The assay demonstrated high sensitivity and specificity, indicating its potential for the monitoring of the effectiveness of immunisation and for the diagnosis of infectious processes. However, the study remained limited in its applicability to the production of rabies immunoglobulins because the purification and stabilisation of the immunoglobulins were not reported. Another of the aspects that influence the efficacy of immunoglobulins as drugs is their physicochemical stability under various environmental conditions. Soliman et al. (2022) have demonstrated the possibility of creating highly effective monoclonal antibodies that will significantly neutralise the rabies virus in laboratory conditions. These findings are of great benefit for the synthesis of new immunobiological drugs but fail to provide a solution for the stability of the polyclonal immunoglobulins that are used in various practical applications. The development of effective immunoglobulin preparations with improved physicochemical stability is hindered by the lack of research on the potential of integrative approaches in the purification and stabilization of immunoglobulins. In terms of efficacy and safety, the TwinrabTM monoclonal antibody cocktail was demonstrated to be a promising alternative to conventional human rabies immunoglobulin in patients at risk of rabies infection (Kansagra et al. 2020). However, this study did not investigate any aspects regarding the production of these monoclonal antibodies.

In the context of the need to develop reliable preparations for the prevention of rabies, it is necessary to consider the methods of purification of immunoglobulins in detail. The aim of this study was to determine the optimal method of purifying anti-rabies immunoglobulins such that the activity of the immunoglobulins is preserved over long periods of storage. The novelty of this study lies in the integrated comparative evaluation of three purification strategies specifically for anti-rabies immunoglobulins obtained from hyperimmunised cattle plasma. Unlike previous studies focused mainly on purification efficiency, this work simultaneously assesses protein recovery, specific IgG activity, virus-neutralising capacity, and short-term storage stability. The objectives of this study were to investigate the methods of precipitation of immunoglobulins using ammonium sulphate, methods of affinity chromatography of the immunoglobulins, methods of combining affinity chromatography with ultrafiltration methods, and the stability of these methods of purification at various storage temperatures.

2. Materials and Methods

2.1 Study design and animals

The study was conducted from January to July 2025 at the Virology Laboratory of the Research and Production Enterprise Antigen LLP, located in Almaty, Kazakhstan. All experimental procedures on animals were performed following the guidelines and recommendations of the European Convention for the Protection of Vertebrate Animals used for experiments and other scientific purposes and were approved by the Institutional Animal Ethics Committee (Approval No. 59-21) (Council of Europe 1986). The study included 30 clinically healthy cattle, randomly divided into three experimental groups (n = 10) based on the method of purification used for obtaining immunoglobulins. The animals were housed under controlled conditions in compliance with veterinary and sanitary requirements at a temperature of 18-22 °C and a relative humidity of 60-70%. They were fed twice a day with a balanced diet.

2.2 Hyperimmunisation and sample collection

Hyperimmunisation was performed with a commercial rabies vaccine (Rabivac, India) administered intramuscularly at a dose of 2 mL, four times at 7-day intervals. On the 28th day after the last injection, 500 mL of blood was collected from the jugular vein of each animal. The blood samples were centrifuged at 3000 rpm for 20 minutes using an Eppendorf 5810 R centrifuge (Germany) to harvest plasma for subsequent immunoglobulin purification. Plasma obtained from each animal was processed individually and was not pooled before purification. Therefore, each animal represented one biological replicate, with ten biological replicates in each purification group. For ELISA, protein concentration measurement, and virus-neutralisation assay, each sample was analysed in triplicate, and the mean value of technical replicates was used for statistical analysis.

2.3. Purification methods

The study used three purification methods – Ammonium sulphate precipitation, Affinity chromatography, and a combined method. In

group I, 50% ammonium sulphate was used for precipitation of plasma proteins, followed by dialysis for the removal of residual salts. In group II, after precipitation, affinity chromatography using a HiTrap Protein G HP column (GE Healthcare, Sweden) was used for purification of immunoglobulins. In group III, a combined approach of ammonium sulphate precipitation, affinity chromatography, and an ultrafiltration step using Amicon Ultra-15 cassettes (Merck Millipore, Germany) was used. For affinity chromatography, elution of bound immunoglobulins was performed with glycine buffer (0.1 M, pH 2.7), followed by neutralisation with Tris-HCl (1 M, pH 9.0).

For each purification procedure, the initial plasma volume, intermediate fraction volume, and final purified fraction volume were recorded. Protein recovery was calculated after each purification step according to the following formula: recovery (%) = (total protein amount after the step / total protein amount in the initial plasma sample) × 100. For Protein G affinity chromatography, the column binding capacity, sample loading volume, equilibration buffer, washing buffer, elution buffer, and flow rate were recorded to ensure reproducibility. Ultrafiltration was performed using Amicon Ultra-15 centrifugal filter units with a molecular-weight cut-off according to the manufacturer's recommendations. SDS-PAGE analysis confirmed differences in the purity profiles of preparations obtained by different purification methods. Preparations purified by ammonium sulphate precipitation contained more visible non-IgG protein bands, whereas affinity chromatography and the combined method showed a more pronounced enrichment of IgG-related bands. The highest relative IgG purity was observed in the combined purification group.

2.4 Determination of protein concentration and antibody activity

Spectrophotometric determination of protein concentration was performed using a NanoDrop 2000 (Thermo Scientific, USA) at a wavelength of 280 nm. Specific anti-rabies IgG antibodies were measured by an indirect ELISA Kit (Creative Diagnostics, USA) according to the manufacturer's instructions.

2.5 Virus neutralisation assay

Biological activity of purified immunoglobulins was assessed by virus neutralisation using a Vero cell line infected with the Challenge Virus Standard (CVS-11) rabies virus strain at a dose of 50 Lethal Dose $(LD)_{50}$. Cytopathic effects were evaluated under a microscope after 72 hours of incubation. Neutralising activity was expressed as the log of the 50% tissue culture infectious dose ($\log_{10}TCID_{50}$) on the basis of inhibition of virus-induced cytopathic effects.

2.6 Stability studies

The stability of purified immunoglobulin preparations was assessed for a 60-day storage period at +4 °C and -20 °C. Changes in protein concentration, antibody titre, and neutralisation activity were measured at 15-day intervals. Potential bacterial or fungal contamination was assessed on Nutrient Agar and Sabouraud Dextrose Agar (HiMedia, India) media, respectively.

2.7 Statistical analysis

The normality of data distribution was verified using the Shapiro-Wilk test. One-way ANOVA with Tukey's test was used to compare the groups, and changes in indicators during storage were analysed using the repeated-measure ANOVA method. The correlation between purification methods and biological activity was assessed using Pearson's correlation coefficient. Differences were considered statistically significant at $p < 0.05$. All data are presented as mean ± standard deviation.

3. Results

3.1 Total protein concentration

Comparative analysis of the three purification methods revealed significant differences in total protein content in anti-rabies preparations. In the ammonium sulphate precipitation group, the lowest concentration (18.81±0.51 mg/mL) was observed, followed by the affinity chromatography method (21.63±0.45 mg/mL). The highest value was recorded in the group with the combined purification approach (25.12±0.65 mg/mL), reflecting the effectiveness of the additional ultrafiltration stage (Table 1). A narrower standard deviation was observed in the affinity chromatography group (0.45 versus 0.51 and 0.65), indicating less variability in protein concentration and, accordingly, higher stability and reproducibility of results. However, in the combined approach group, the value was 33.5% higher than the ammonium sulphate precipitation group and 16.1% higher than the affinity chromatography group.

Table 1. Concentration of total protein in anti-rabies preparations after various purification methods

Purification method	Total protein concentration, means ± SD (mg/mL)
Ammonium sulphate precipitation	18.81 ± 0.51 [c]
Affinity chromatography	21.63 ± 0.45 [b]
Combined method	25.12 ± 0.65 [a]

Different superscript letters indicate statistically significant differences between groups according to Tukey's post hoc test, $p < 0.05$; SD: Standard deviation

The data presented in Table 1 show that the purification method had a clear effect on the total protein concentration in anti-rabies preparations. The combined purification method led to the highest protein concentration of 25.12 ± 0.65 mg/mL, followed by affinity chromatography (21.63 ± 0.45 mg/mL). The lowest total protein concentration was determined by the precipitation method (18.81 ± 0.51 mg/mL). The results of the lower protein concentrations obtained by the precipitation method suggests the greater effectiveness of the combined purification method. The improved protein concentrations obtained using the combined purification method may be due to the additional step of ultrafiltration, which concentrated the immunoglobulins and removed the low-molecular-weight components from the fraction. The lower protein concentration prepared using the precipitation method may be due to the low selectivity of the process or the loss of the proteins during the precipitation and dialysis steps. These results indicate that the combined purification method provided the best yield of the proteins compared to the other methods described. Although the total protein concentration is an indicator of the recovery and concentration of the proteins, it is not an indicator of the purity of the IgG. Furthermore, the total protein also contains other plasma proteins in addition to the IgG. Therefore, the protein concentration value must also be evaluated in conjunction with the anti-rabies IgG activity and virus-neutralising activity.

3.2 Specific IgG activity

The specific anti-rabies activity of the immunoglobulins assessed by an indirect ELISA method indicated a marked dependence of IgG titres on the purification method used (Table 2). The ammonium sulphate precipitation group yielded the lowest IgG titre (0.861 ± 0.047),

indicating a partial loss of functionally active immunoglobulins during the isolation process. Significantly improved IgG titres were observed in the affinity chromatography group (1.109±0.041), indicating significant preservation of functionally active antibodies. An even more pronounced effect was observed in a combined purification approach (1.376±0.046), reflecting both the effectiveness of removing unwanted impurities and the concentration of target molecules.

Table 2. Titres of anti-rabies IgG in preparations after various purification methods

Purification method	IgG titre, means ± SD (OD)
Ammonium sulphate precipitation	0.861 ± 0.047 [c]
Affinity chromatography	1.109 ± 0.041 [b]
Combined method	1.376 ± 0.046 [a]

Different superscript letters indicate statistically significant differences between groups according to Tukey's post hoc test, $p < 0.05$; SD: Standard deviation; OD: Optical density

The data presented in Table 2 demonstrate that the purification method influenced the preservation of specific anti-rabies IgG activities. The combined purification method showed the highest IgG titre (1.376 ± 0.046 OD), indicating the greatest retention of antigen-binding immunoglobulins after processing. Affinity chromatography produced an intermediate value (1.109 ± 0.041 OD), which suggests that selective binding to the Protein G column helped preserve functionally active IgG more effectively than precipitation alone. The lowest IgG titre was observed after ammonium sulphate precipitation (0.861 ± 0.047 OD), probably due to partial antibody loss or structural alteration during salt precipitation and subsequent dialysis. Overall, these results indicate that the combined purification protocol improved not only the quantitative recovery of immunoglobulins but also their specific antigen-binding activity.

3.3 Virus-neutralisation activity

The biological activity of anti-rabies immunoglobulin preparations was assessed by the virus neutralisation method using a Vero cell line infected with the CVS-11 rabies virus strain. The results showed marked differences in virus-neutralising ability between preparations purified by different methods. The minimum level of activity was recorded in the group with ammonium sulphate precipitation: the average neutralising capacity was 1.79±0.20 logarithm of the tissue culture infectious dose 50% ($log_{10}TCID_{50}$), which reflects the insufficient preservation of the functional activity of antibodies with this purification method. In the group where affinity chromatography was used, this indicator increased to 2.28±0.10 $log_{10}TCID_{50}$, indicating better preservation of the biologically active IgG fraction. The highest virus-neutralising activity was observed with combined purification: 2.87±0.08 $log_{10}TCID_{50}$, indicating a high degree of preservation and concentration of functionally active immunoglobulins (Table 3).

Table 3. Virus-neutralising (VN) activity of anti-rabies preparations

Purification method	VN activity, means ± SD ($log_{10}TCID_{50}$)
Ammonium sulphate precipitation	1.79 ± 0.20 [c]
Affinity chromatography	2.28 ± 0.10 [b]
Combined method	2.87 ± 0.08 [a]

Different superscript letters indicate statistically significant differences between groups according to Tukey's post hoc test, $p < 0.05$; SD: Standard deviation; TCID: Tissue culture infectious dose

An assessment of the stability of anti-rabies immunoglobulins conducted over 60 days at temperatures of +4 °C and -20 °C showed a clear dependence of the preservation of the functional and physicochemical characteristics of the preparations on the selected purification method and storage conditions. In general, at a temperature of +4 °C, a gradual degradation of the main indicators was observed in all groups: the concentration of total protein, specific IgG titres, and virus-neutralising activity decreased. These changes became particularly noticeable after 30 days of storage. In contrast, when frozen at -20 °C, all the parameters studied were preserved significantly better, showing only minor fluctuations. Preparations obtained using a combined purification method showed greater resistance to parameter changes at both temperature regimes, while immunoglobulins purified with ammonium sulphate were characterised by the least stability. This confirms the importance of both the purification method and storage conditions for maintaining the therapeutic activity of anti-rabies preparations throughout their shelf life (Table 4).

As presented in Table 4, the temperature at which the anti-rabies immunoglobulin was stored had a considerable effect on the stability of the product after 60 days of storage. Each of the groups of immunoglobulins that were purified according to the different methods exhibited a decrease in the measured concentrations of the total protein, IgG, and virus-neutralising activity after storage at +4 °C. The most notable decreases in activity were seen in the samples that were prepared using the method of precipitation with ammonium sulphate, which had measured levels of 17.48 mg/mL of total protein, 0.791 OD of IgG titre, and 1.66 $log_{10}TCID_{50}$ of virus-neutralising activity after storage at +4 °C for 60 days. The preparations that were purified using affinity chromatography had better preservation of the parameters that were studied, especially at -20 °C. The results of the analysis of the samples that were purified using the combined purification method exhibited the best preservation of the studied parameters. After storage for 60 days at -20 °C, the combined purification method resulted in 24.92 mg/mL of total protein, 1.341 OD of IgG titre, and 2.85 $log_{10}TCID_{50}$ of virus-neutralising activity. These results indicate that the combination of precipitation, affinity chromatography, and ultrafiltration provided the greatest resistance to storage-related changes. Overall, Table 4 confirms that the combined purification method together with frozen storage at -20 °C was the most effective approach for maintaining the quality and biological activity of anti-rabies immunoglobulin preparations.

4. Discussion

The quantitative analysis of the total protein content in anti-rabies preparations obtained by the three different methods of protein purification in this study revealed significant differences in the concentrations between the methods. The precipitation of proteins with ammonium sulphate at 50% saturation was demonstrated as the least effective method, yielding an average protein concentration of 18.81±0.51 mg/mL. Although it is a simple and cost-effective method of protein purification, its lack of selectivity potentially leads to the loss of proteins during the purification protocol. Furthermore, the high ionic strength of the solution used in this method may result in the partial aggregation and denaturation of the proteins, particularly under non-optimal conditions of the solution pH and temperature. The reduction in the protein concentration using this protocol is explained by the literature that reports the co-precipitation of unwanted protein fractions with this method as well as the instability of the immunoglobulin G

Table 4. Dynamics of changes in the characteristics of anti-rabies preparations during storage

Purification method	Temperature	Storage day	Protein concentration (mg/mL)	IgG titre (OD)	VN activity ($log_{10}TCID_{50}$)
Ammonium sulphate	+4 °C	60	17.48	0.791	1.66
Affinity chromatography	+4 °C	60	20.23	1.022	2.07
Combined method	+4 °C	60	23.77	1.279	2.67
Ammonium sulphate	-20 °C	60	18.29	0.855	1.72
Affinity chromatography	-20 °C	60	21.44	1.101	2.26
Combined method	-20 °C	60	24.92	1.341	2.85

OD: Optical density (at 450 nm); VN: Virus-neutralising activity; TCID: Tissue culture infectious dose

structures when they are redissolved (Fan et al. 2022; Zhai et al. 2022). Thus, loss of proteins during the precipitation step is both quantitative and qualitative. The affinity chromatography method using the HiTrap Protein G HP column proved to be more efficient, as it yielded an average concentration of 21.63±0.45 mg/mL. The main advantage of this technique lies in the fact that the Fc fragments of immunoglobulin G have a high affinity for the immobilised ligand, which leads to increased levels of purity and preserved properties of the protein. The standard deviation of the result of this method was lower than the other groups (0.45 vs 0.51 and 0.65), signalling the higher stability and reproducibility of this technique. Additionally, due to the properties of this technology, the probability of protein loss is lower. The elution step occurs at specific pH levels, temperatures, and lengths of contact with the acidic environment. By neutralising the acid in the buffer, the immunoglobulins will not be denatured. Several previous studies have already demonstrated that this technology is highly reproducible and compatible with other technologies (De Melo et al. 2022; Wu et al. 2024). The results presented in this study reaffirm those findings.

The highest values of protein concentration were obtained by the group that underwent purification of immunoglobulins using all three described methods – precipitation, affinity chromatography, and ultrafiltration. The average protein concentration value in this group was 25.12±0.65 mg/mL, which is 33.5% higher than the ammonium sulphate precipitation method alone and 16.1% higher than the affinity chromatography method alone. Ultrafiltration allowed for the immunoglobulin solution to be concentrated without the use of chemical reagents and further purified the solution of immunoglobulins by removing components of low molecular weight from the solution. Each of the methods compensates for the shortcomings of the others. Precipitation allows for the initial concentration of immunoglobulin-rich fraction; affinity chromatography removes the residual non-IgG protein impurities and enrich the target IgG fraction; and ultrafiltration removes any remaining impurities of low molecular weight from the immunoglobulin solution. The results of the research are in accordance with the research of Lupitha et al. (2025) and Bartaquini et al. (2020). Furthermore, one-way ANOVA analysis of the results from the three groups indicated significant differences in protein concentration between the groups ($p < 0.05$).

The data obtained using ELISA assays indicated the differences in the activity of IgG-specific components between the tested groups, which is also reflected in the results of the measurement of the concentration of the proteins (Chorpunkul et al. 2024). In the first group of samples, which were precipitated with ammonium sulphate, the average value of IgG activity was determined to be 0.861±0.047 OD. The use of this method not only contributed to the ineffective precipitation of the target protein, but may have also led to the denaturation of the immunoglobulins. The dialysis step that was used to remove the ammonium sulphate residues from the protein solution may have also contributed to the loss of protein and its activity. High salt concentrations are known to contribute to the destabilisation of the tertiary and quaternary structures of proteins, which has been demonstrated by authors Lumlertdacha et al. (2023) and Hou et al. (2024). The decrease in the specific activity of the preparation in this case is, therefore, unacceptable for the preparation of immunobiological preparations. In the second group, treated using affinity chromatography, the average specific IgG titre increased to 1.109±0.041 OD. The highly selective nature of this method allows for the preservation of the spatial structure of the antibodies. The standard deviation in this group was the lowest of all groups, indicating the high reproducibility of this method. These features of the affinity chromatography method were highlighted in studies by Zhang et al. (2022) and Santosh et al. (2024). However, other studies, such as that by Yumoto et al. (2021) suggest that the use of affinity chromatography is not advantageous over the use of methods like precipitation. However, the results of this study suggest that the affinity chromatography method is highly reliable, as evidenced by the homogeneity and high IgG titres within the products of this group.

The highest specific activity values were recorded in the group that underwent purification through the combined method. The IgG titre of this group was 1.376±0.046 OD, which is higher than the titre values of the other groups. The ultrafiltration performed at the last stage of purification effectively removed the compounds of low-molecular weight from the preparation of IgG, which significantly decreased the amount of ballast compounds such as buffer and denatured IgG that were present prior to purification. The result of the ELISA of the purified IgG preparation demonstrates that the preparation has high specific activity, as the higher optical density of IgG in this preparation indicates the saturation of the available binding sites of the ELISA wells with IgG of high biological activity. The conclusions of both Wu et al. (2025) and Manalo et al. (2020) that the removal of low-molecular weight compounds from IgG preparations can significantly increase the specific activity of the preparations are confirmed by both the optical density measurements of IgG from each preparation, as well as the consistent data of each of these measurements. Beyond the removal of compounds that may interfere with the biological activity of IgG preparations, the various stages of preparation have helped preserve the biological activity of the IgG. The IgG molecule is stable during the various preparation steps, as the conditions are gentle enough to not denature the antibody or alter its epitope. While IgG preparation methods, utilizing multi-stage purification protocols, can lead to instability of the IgG and the concerns could be warranted on an

industrial scale preparation of IgG (Cai et al. 2023; Zorzan et al. 2023). However, the data obtained in this preparation protocol confirms the IgG stability and high level of efficiency of the method. The specific IgG activity differed significantly between the groups ($p < 0.05$).

The results of the virus neutralisation test (using the Vero cell line and the CVS-11 strain of rabies virus) help to further confirm the correlation between the purification method and biological activity. The least pronounced ability of the virus to be neutralised was exhibited by samples that were purified using ammonium sulphate precipitation; these samples had an average activity of 1.79±0.20 $\log_{10}TCID_{50}$, which is a very low value compared to the required value of >2.0 $\log_{10}TCID_{50}$ (Mo et al. 2025). Furthermore, the high value for the standard deviation (0.20) indicates that the technological reproducibility of this process is poor – there are many factors that may have contributed to the low activity of the purified samples, such as the precipitation process itself; the stability of the antibodies in high levels of salt; and the dialysis step that occurred after precipitation to remove the ammonium sulphate, all of which could have led to denaturation of the active immunoglobulin G (IgG) sites of the antibodies (Liu et al. 2023). The results of affinity chromatography were significantly better, achieving an average virus-neutralising activity of 2.28±0.10 $\log_{10}TCID_{50}$. The method allows for the isolation of the fraction of immunoglobulins that demonstrates biological activity, maintaining the spatial structure of the proteins and preventing their inactivation – properties that Ejemel et al. (2022) and Quiambao et al. (2024) have indicated as acceptable for the immunoglobulins in question. Furthermore, the lower standard deviation indicates that the products are more homogenous and stable. However, the presence of some ballast proteins or factors that reduce the activity of the preparation may limit the effectiveness of this method compared with the combined method.

The best results were seen in the group that used the combined purification process. The virus-neutralising activity index recorded by this group was 2.87±0.08 $\log_{10}TCID_{50}$, which is the optimal value for this parameter. This result was achieved due to the ability of ultrafiltration to remove low-molecular-weight inhibitors of the virus, while also increasing the concentration of the target IgG molecules. Furthermore, the low standard deviation of this outcome indicates the degree of standardisation of this technology. The preparations of this group exhibited rapid and stable neutralisation of the virus without any cytopathic effects. A one-way analysis of variance test indicated that there are significant differences between the three groups ($p < 0.05$). Although Chorpunkul et al. (2024) state that any complicated technological scheme poses a threat of the loss of active components of the preparation, the outcome of this study indicates that using the combination of purification methods that were optimised in advance can mitigate these risks, while also increasing the yield of the desired active product. The results of a 60-day stability study show that the effectiveness of preserving the physicochemical and biological properties of the preparations depends on both the purification method and the temperature regime. The mean values of all three parameters declined at +4 °C, particularly after the 30-day storage period, indicating slow but active degradation processes occurring during storage resulting in the loss of the biological activity of the preparations. The most pronounced decline in biological activity at +4 °C storage was experienced in preparations purified with ammonium sulphate precipitation method.

Due to the removal of unstable and low-molecular-weight protein impurities, better storage stability at +4 °C of purified preparations was demonstrated by affinity chromatography, resulting in greater structural homogeneity and resistance to denaturation. However, after 60 days of storage, a moderate decline in mean values of all indicators was observed, particularly in virus-neutralising activity, suggesting the unsuitability of long-term storage at +4 °C irrespective of the purification method used. The preparations that experienced combined purification, including the ultrafiltration stage, exhibited the highest level of stability. These preparations contained the highest concentration of the active component, which was stable throughout the storage period (at +4 °C), with minimal degradation of the active component occurring during that storage period. Within the group of preparations that experienced these various purification steps, the most pronounced advantages in relation to stability were experienced with storage at -20°C, where all preparations within the group experienced minimal fluctuation in their initial values of each of the three parameters. Pati et al. (2023) reported similar results for purified anti-rabies IgGs, indicating the importance of both the purification and storage of the preparation to ensure its stability over time. Additionally, Rathnadiwakara et al. (2025) produced similar results for anti-rabies IgG preparations but also noted that both humoral and cellular immune responses should be considered when producing an anti-rabies immunoglobulin preparation, again indicating the importance of the preparation's overall comprehensiveness in the context of rabies prophylaxis, though in relation to the immunogenicity of a rabies vaccine rather than the technological aspects of the preparation of IgG immunoglobulins. The differences between the various groups of samples were found to be statistically significant through analysis of variance ($p < 0.05$).

Thus, the study demonstrated that the purification method has a critical impact on the physicochemical and biological characteristics of anti-rabies immunoglobulins. The combined technology, which integrates precipitation, affinity chromatography, and ultrafiltration, provides the highest yield of the target protein, its specific activity and virus-neutralising capacity, as well as high stability during storage. The optimal stability of anti-rabies immunoglobulins is achieved by combining deep purification with storage at -20°C, ensuring the preservation of clinical efficacy and safety of use throughout the shelf life. The data obtained can be used to recommend this approach as the most preferable for the development and standardisation of anti-rabies preparations. However, the current study has certain limitations. First, the experiment was conducted under laboratory conditions, which may not fully reflect the variability of large-scale production or field storage. Second, the virus-neutralisation assay was based on a single rabies virus model, limiting the generalisability of the findings to other strains. Third, the evaluation focused mainly on protein concentration, IgG titre, neutralising activity, and short-term stability. Further studies should include broader preclinical testing, longer storage periods, immunogenicity assessment, toxicological evaluation, and validation under real logistical conditions.

4. Conclusions

The study demonstrated that the purification method substantially influenced the quality and stability of anti-rabies immunoglobulin preparations. Ammonium sulphate precipitation was the least effective approach, as it resulted in lower protein recovery and reduced preservation of functionally active antibodies. Affinity chromatography improved the selectivity and biological activity of the preparations by

better preserving the IgG fraction. The combined method, which included precipitation, Protein G affinity chromatography, and ultrafiltration, showed the best overall performance across all assessed parameters. It provided better protein preservation, higher specific anti-rabies activity, stronger virus-neutralising capacity, and greater stability during storage, particularly under frozen conditions. These findings support the potential use of the combined purification protocol as a promising technological approach for producing stable and biologically active anti-rabies immunoglobulin preparations.

Declarations

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