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An optimized method of IgG's precipitation with ammonium sulphate from hyper immune horse plasma for snake anti venom production

Abstract

The treatment of choice for the snake bite is the administration IgG antibodies that are raised in an animal body thorough immunization of the animals. To avoid potential adverse reactions that arises from introduction of animal antiserum in human body, the antibody needs to be purified from the other non-specific proteins like albumin. Optimal conditions for the fractionation of IgG's were studied; ammonium sulfate was added to plasma up to 40% concentration to separate non-specific proteins, iso electric separation was carried out at pH 5.4, and the final IgG's fractionation by the addition of ammonium sulfate up to 50% concentration. A highly purified and concentrated mixture of IgG's was obtained after removal of ammonium sulfate through dialysis. The resulting anti venom was of good quality in terms of purity and was found to be highly potent against venoms of all four types of snakes. Owing to the high purity and greater potency of the anti venom the method has potential to be used for lab and commercial scale production of anti venom.

Keywords: snake anti venom, passive immunity, immunization, horse, antibodies, ammonium sulfate fractionation

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Introduction

Artificially acquired passive immunity is a form of immunity in which antibodies are directly transferred into the body of affected individual to achieve rapid neutralization of antigens or to treat such individuals in which antibodies cannot be developed otherwise such as immunodeficiency diseased or hypogammaglobulinemia.[1] There are some diseases against which vaccine and chemical drugs cannot be synthesized. So, introduction of antibodies into body remains the only feasible option to treat such diseases. This is also a treatment of choice for the treatment of poisoning and envenomation. Passive immunity provides a short-term but rapid immunity to the individuals.[2] Snake envenoming is one of such diseases that is prevalent throughout the world, mostly in the Africa, South America and south Asian countries and treated with anti-snake venom serum.[3] According to WHO estimates of 2018 approximately 5.4 million people receive snake bites every year with up to 2.7 million cases of envenoming, and about 81, 000 to 138, 000 deaths occur due to these bites.[4]

Antiserum treatment against the snake venom is the preferred treatment in case of snake envenoming in which antisera is raised against venom in animals.[5] In this method the animal i.e. horses, sheep, camels etc. are immunized against the snake venom by injecting appropriate doses of snake venom into the animal body. The animal body produces antibodies against that venom.[6] The venom doses are increased later on to get hyper immune blood to harvest maximum amount of IgG's. The animals are then bled, and the required antibodies (IgG's) are fractionated from the plasma. Since antibodies are the proteins, so it needs to be fractionated from other plasma proteins. To precipitate dissolved proteins the principal strategy is to reduce number of hydrogen bonds between the dissolved protein and solvent molecules.[7] This can be efficiently done by the addition of salt in the plasma. The salt molecules remove the water molecules from the protein and thus the number of hydrogen bonds between protein and water decreases, resultantly the protein is precipitated. The solubility of protein depends upon the ionic strength of the solution and hence the concentration of salt. Actually, low salt concentration favors the solubility of protein a phenomenon known as 'salting in'. As the salt concentration increases the solubility of protein decreases. Ultimately at very high concentration salt will force out the protein to precipitate by the phenomena of 'salting out'.[8] Since proteins differ in their solubilities according to ionic strength of solution, salting out can be very useful to precipitate required protein.

Several methods are available for the precipitation of antibodies which includes caprylic acid fractionation,[9] ethanol precipitation,[10] affinity and ion exchange chromatography.[11] But a very easy, cheap and more popular technique of antibody separation is salt precipitation.[12] This technique involves use of sodium or ammonium sulfate for the precipitation on immune globulins.[13] This technique is especially useful for large scale precipitation of immune globulins for commercial purposes.[14]

Materials and methods

Venom collection

The study was approved by the Institutional Ethical Review Board of National Institute of Health Islamabad. Venom of 'big four' snakes Cobra (*Najanaja*), Common krait (*Bungarus caeruleus*), Russell's viper (*Viperarusselii*), Saw-scaled viper (*Echiscarinatus*) were collected at Biological Production Division, National Institute of Health Islamabad, Pakistan.

Immunization of horses

Four large adult horses of local breed were taken aged more than 5 years. Each horse was immunized with the sterile liquid preparation of venom of Cobra, Krait, Saw Scaled Viper and Russel viper

separately. Immunization was started with 10µg of venom of Cobra & Russel's viper, 5µg of venom for Echis & 1.0µg venom for Krait. The horses were hyper immunized with venoms for three months up to approximately 30mg venom of each snake. After completion of immunization, the horses were checked for antibody titer. After which a total of 10-liter blood from each horse were drawn in two bleedings with the addition of potassium oxalate as anticoagulant. The plasma was separated through centrifugation and storedat 4-8°C.

Ammonium sulfate precipitation

Saturated ammonium sulfate solution was prepared one day before processing. The 50ml aliquots of plasma was diluted 50% with WFI and the ammonium sulfate solution was then added at concentration of 20%, 30%, 40%, 50%, 60% and 70%. The filtrate and precipitates were tested for albumin and globulin contents and an optimized concentration was picked for separation of non-specific serum proteins like albumin and second concentration value was picked for extraction of specific proteins i.e., IgG's. Theses concentration values was then used for the development of final process.

The plasma was first diluted with distilled water. Sodium pyrophosphate, toluene and calcium chloride were added at a concentration of 2g/L, 1.5g/L and 0.04% (40g/L) respectively with continuous stirring. Then ammonium sulfate was added up to concentration of 40% and the mixture was filtered. Then the pH of the solution was raised to 5.4 and stirred thoroughly at 30°C at room temperature for half an hour. The precipitate was discarded and ammonium sulfate was added again up to concentration of 50% in the filtrate and then filtered. The filtrate was discarded and precipitate was separated out. The precipitate was dialyzed through a dialyzing membrane to remove remaining ammonium sulfate. The removal of ammonium sulfate was confirmed through barium chloride test. The pH of the mixture was then raised up to 7.00. This mixture contains required immunoglobulins (IgG's). The mixture is stored at 4-8°C and the potency of mixture was checked according to LD_{50} and $2LD_{50}$ and the subsequent dilutions of anti-venom were made accordingly.

Check for removal of ammonium sulfate

Barium chloride test is used for the detection of removal of ammonium sulfate. 5% solution of barium chloride is prepared and 1ml of it is added to 1ml dialysate. The presence of turbidity will indicate the presence of ammonium sulfate while a clear solution indicates that the ammonium sulfate is removed.

Potency check

The potency of the mixture was checked against the LD_{50} and $2LD_{50}$ of 'big four' snakes calculated by Parveen et al.[15] Healthy male mice of weight 18-20g were taken and 1:10, 1:20, 1:30, 1:40 and 1:50 times dilution of the anti-snake venom was mixed with venom to check for the neutralization potency of the anti-snake venom. A dose of 0.5ml of mixture of venom and anti venom was injected through intra-caudal route. The result was checked after 24 hours as mortality and survival rate of the mice. The anti-snake venom was then diluted with normal saline according to the results of potency.

Total proteins and albumin estimation

Finally, the diluted anti-venom serum was checked for the estimation of total proteins, albumin, globulins and nitrogen contents. Albumin is estimated by 'spectrum albumin-BCG (acetate buffer) kit and total proteins was estimated by 'spectrum total protein biuret reagent' kit.

Results

Plasma processing

The horses have different amount of plasma in their blood. Roughly 5 liters plasma was obtained from 10-liter whole blood using potassium oxalate as anticoagulant. Addition of ammonium sulfate up to 40% concentration turns the solution milky that is an indication of protein precipitation. The filtration results in the white precipitates of non-specific proteins and a clear solution that contains the specific proteins. After second addition of ammonium sulfate, concentration of ammonium sulphate causes the precipitation of specific proteins (IgG's). The precipitates were filtered to separate pure IgGs and filtrate. Additionally, the pH 5.4 at iso electric step also causes the precipitation of non-specific protein. The residual ammonium sulfate was removed in the dialysis step. The removal of ammonium sulfate was confirmed through barium chloride test. After dialysis the precipitate turns in to a thick greyish black solution which is the final product.

Finding the optimum concentration values

Table 1 shows the concentration of albumin, globulins and total proteins contents of the precipitates at different concentration set of solution. The results clearly indicate that the albumin is best precipitated at the concentration of 40% and the globulins are best precipitated at the concentration of 50%. So, 40% concentration of ammonium sulphate in plasma solution was chosen to precipitate non-specific albumin proteins and the 50% concentration was chosen to precipitate IgGs from the hyper immunized horse plasma.

Table 1 Concentration of albumin, globulins and total proteins contents at different concentrations of ammonium sulphate solution

Concentration (%)	Albumin concentration (g/dl)	Globulin concentration (g/dl)	Total proteins (g/dl)
20	4.2	0.31	8.5
30	5.7	0.54	8.7
40	7.2	0.97	8.5
50	2.9	2.44	8.5
60	2.6	2.1	8.6
70	2.5	1.93	8.5

Potency test of the antivenom

The fractioned IgGs were subjected to animal testing for potency test of anti-sera. Table 2 shows the results of different dilutions of anti-snake venom serum against challenge dose of $2LD_{50}$ of each venom. The anti-venom was potent to neutralize $2LD_{50}$ for up to 50 times dilution. Complete neutralization was observed up till 40 times dilution. So, the anti-venom was diluted 40 times to observe the results of final product.

Albumin, globulin and total proteins contents of final product

Table 3 shows the total proteins, albumin and globulin contents of the finally diluted anti-snake venom serum which is fractionated with above mentioned ammonium sulfate precipitation procedure.

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Table 2 Potency test results of anti venom on mice

Dilution of sample anti venom	Mice injected	Cobra venom(% mortality)	Russel's viper venom(% mortality)	Saw scaled viper venom(% mortality)	Krait venom(% mortality)
01:10	4	0	0	0	0
01:20	4	0	0	0	0
01:30	4	0	0	0	0
01:40	4	0	0	0	0
01:50	4	25	50	25	50
+ve control LD_{50}	4	50	50	50	50
+ve control $2LD_{50}$	4	100	100	100	100
-ve control (PBS)	4	0	0	0	0

Table 3 Total protein, albumin, globulin, total proteins and nitrogen contents (g/dl) in the final product

Test	Results
Total proteins	2.81
Albumin	0.284
Nitrogen	0.449
Globulin	2.503

Discussion

Protein fractionation by salts is commonly used practice for the precipitation of protein from various sources including plasma. Ammonium sulphate and sodium sulphate salts are frequently used for this purpose. Ammonium sulphate is preferred due to its feasible cost and easy availability. Its long been used experimentally and commercially for the fractionation of proteins because it also stabilizes the protein structure.[16] The purity and yield of anti venom produced by this procedure are also high, so it induces fewer allergic reactions upon its injection into the human body.[5] Its molecular mechanism is also well understood. The basic principle behind salt precipitation is that the salts enhance the solubility of the proteins at low concentrations up to i.e.<0.5M a phenomenon known as 'salting in'. Further increase in salt concentration led to decrease in protein solubility until the proteins are precipitated out which is called as 'salting out'.[16] When the saturated solution of ammonium sulphate is added to plasma the major nonspecific protein of serum which is albumin protein is precipitated out due to its low molecular weight i.e. 66 kDA. Additionally, the 5.4 pH of the solution causes the further precipitation of clotting proteins present in plasma. The filtrate after separation of these non-specific proteins was again processed with 50% ammonium sulphate concentration solution. At this high concentration of ammonium sulphate the high molecular weight bovine IgGs (M.W~150 kDA) are precipitated out.

The data clearly indicate that the conditions described in this procedure is quite feasible to fractionate IgG's from the horse serum. The 40% concentration of ammonium sulphate and pH 5.4almost completely remove albumin and other non-specific proteins from the plasma as a result highly pure anti-serum is obtained. This procedure also precipitate pure IgGs at ammonium sulphate concentration of 50% which make further processing of the anti venom easier. These optimal conditions are easy to adopt and manipulate under laboratory conditions and can also be employed to commercial level.

The higher purity, better neutralization ability and easy manipulation of the anti venom produced by ammonium sulfate method make this a popular method of choice worldwide for the production of anti venoms against various different venomous organisms. The simplicity of this method also makes it a preferable method because it does not require very sophisticated equipment for its operation. However, the ammonium sulfate technique is considered old, less efficient and time consuming. The world is now switching to more efficient and less time-consuming methods for the production of anti venoms. Many of such methods like caprylic acid fractionation method, salt precipitation, affinity and ion exchange chromatography etc. have been successfully tested under laboratory conditions and some of them are also adopted for commercial purposes but the ammonium sulfate method is still most popular method for the precipitation of proteins and production of anti venoms across the world.

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Statement of conflicts of interest

The authors declare no conflict of interest.

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