International Journal of Natural Medicine and Health Sciences
ISSN (Online):2790-2471 ISSN (Print): 2790-2463
Volume 2(1) December 2022
Journal homepage: https://journals.iub.edu.pk/index.php/ijnms

Review

A comprehensive review on the preparation of pure immunoglobulins

Muzzamil Shehzad[a*], Tayyab Ali[b], Nusrat Bibi[c], Shoaib Shafique[d]

[a*] Faisalabad Institute of Research Science and Technology, University of Health Sciences, Lahore, Pakistan
[b] Clinico-Molecular Biochemistry Laboratory, Department of Biochemistry, University of Agriculture, 38000, Faisalabad, Pakistan
[c] Department of Microbiology, Government College University Faisalabad, Pakistan
[d] School of Biochemistry and Biotechnology (SBB), University of the Punjab Lahore, Pakistan

Article Info.

Received: 25-11-2022
Revised: 28-12-2022
Accepted: 28-12-2022
Online: 31-12-2022

Correspondence:
muzzamilshehzad8@gmail.com

Keywords: Antibodies, Chromatography, Electrophoresis, Immuno-adsorbent, Immunoglobulins.

Abstract

Immunoglobulins are also known as antibodies. Plasma cells are responsible for the production of immunoglobulins. Beta cells are activated against a pathogenic attack and facilitated the formation of different types of immunoglobulins naturally. These immunoglobulins are also artificially synthesized by non-specific laboratorial techniques include fractionation precipitation, electrophoretic methods, gel filtration chromatography, ion exchange chromatography, hydrophobic chromatography and by the specific immuno-adsorbent methods. Third generation immunoglobulins are effectively used for therapeutic purpose against viral infections intravenously. Second generation immunoglobulins synthesis involved removal of anti-complement contaminants and IgG aggregates, through enzymatic degradation and chemical modification. The harmful effects of intravenous immunoglobulins can be reduced by using ultraviolet light, P-propiolactone, and pepsin at pH 4. Intravenous immunoglobulin drugs lead to anti-inflammatory and immunomodulatory effects in various infections. The mechanism of action of intravenous immunoglobulins is dependent on the binding between the Fc portion of injected IgG and target cell receptors. Radiolabeling is performed by two methods as in vivo, in which radiolabeled antibodies are incorporated into the body to bind with the antibodies and in vitro method, radioactive material is bound with already formed antibodies. This chapter highlighted the artificial methods adopted for production of radiolabeled immunoglobulins holding significant therapeutic and diagnostic applications.

Citation: Shehzad M, Ali T, Bibi N, Shafique S. A Comprehensive Review on Preparation of Pure Immunoglobulins. IJNMS. 2022; 2(1)48-55.

Introduction: This review includes the preparation of pure immunoglobulins. There are mainly two basic ways by which immunoglobulins can be prepared, namely, specific and non-specific methods. These includes the use of electrophoretic methods, chromatographic methods and many more. The method or technique that was used for the preparation of immunoglobulins mainly relies on the specific type of immunoglobulins. As some immunoglobulins are cellularly synthesized while other are synthesized non-cellularly. After the preparation, there are some old techniques in which purification of these immunoglobulins is carried out as an essential step. So, purification step also enhances the efficacy of the respective immunoglobulins. In some techniques for the preparation of immunoglobulins, binding of ligands is necessary. Nowadays, labelling of immunoglobulins is very essential because these antibodies can be widely used in the preparation of ELISA-based fluorescence diagnostic kits and used to optimise the radio-immuno assay protocols.

Preparation of Pure Immunoglobulins: Immunoglobulins (Ig) also known as antibodies exists in serum. Plasma cells are responsible for antibodies or glycoproteins production. When particular immunogens such as bacteria and viruses enter into the body, β cells are activated according to specific immunogens. These cells synthesize the proteins which are involved in humoral immune responses against chemicals, microbes and synthetic substances.

Non-specific methods: Fractionation precipitation: The solubility of proteins depends upon its solubility envelop. Protein molecules are surrounded by a variety of other molecules such as polysaccharides, nucleic acids, polyphenols, pigments, cationic surfactants, and ionic surfactants. These molecules are eliminated from the blood by adding different neutral salts (ammonium sulphide, sodium chloride, and sodium sulphide). The solubility of proteins decreases with increasing salt concentration [1]. At very high salt concentration, proteins become insoluble and precipitates. Protein structures do not denature using ammonium salts and have no adverse effects on enzyme activity. Immunoglobulins are usually precipitated by using sodium sulfate and ammonium sulfate. Precipitation fraction of IgG required pH 7.3 at room temperature; 33% concentration is achieved by adding ammonium sulfate into serum and finally, 18% concentration is accomplished by adding sodium sulfate. Proteins after precipitation are added into phosphate-buffered saline (PBS) and centrifuged. About 12-15% Na_2S0_4 or 33% $(NH4)_2S0_4$ are added again for precipitation. This process is repeated again and again according to the requirements. To obtain pure IgG from rabbit and human serum, PBS is added and filtration is performed using the Sephadex G-25 chromatographic column to remove extra salts. Sometimes, different salts are usually used for pure IgG isolation from chickens and mammal's serum. Ammonium sulfate is used for IgG isolation from the serum of guinea pig to get 40% saturation. About 40-50% saturation of immunoglobulins is achieved using ammonium sulfate at pH 7.0. The immunoglobulins after precipitation are segregated by centrifugation and again dissolved in PBS. The euglobulin fraction of immunoglobulin is precipitated for storage. 50-60% of Ammonium sulfate used for the preparation of main IgM as shown in fig (1) [2].

Potassium phosphate buffer in 60 mM concentration is added in antiserum (20 mL) for dilution and incubated at 4°C, at pH 7.0 for 24 hours. Then centrifuged after incubation to remove precipitates and discarded. Supernatant 13% concentration is maintained by adding polyethylene glycol (PEG) 6000 (BDH). Centrifugation is performed for final precipitates and potassium phosphate buffer at 30 mM concentration was mixed at pH 7.0. PEG 13% again was added to this solution and resulting precipitates were in potassium acetate solution (30 mM) at pH 4.6. Again, 10% PEG was added and centrifuged to remove precipitates. Solution of NaOH (2N) was added to maintain 5.8 pH of supernatant, then shaked for 30 min and centrifuged to remove precipitates. PBS 20 mL was added and dialyzed against PBS. IgM is obtained at 7% saturation and 1gA at 14% saturation. Deionized water 57 mL was used to dilute 19 mL of antiserum by adjusting pH to 7.7. Then cool down at 0°C. 20% final concentration was achieved by adding 50% ethanol and reducing the temperature to -5°C. Centrifugation at -5°C removed resulting precipitates and 15 mM solution of NaC1 was added. 50 mM acetic acid was used to adjust the pH to 5.2 and temperature below 0°C. IgM and IgA immunoglobulins were present in precipitated material. Centrifuged the material for protein removal. The pH was increased to 7.4 by adding a solution of 0.5 M Na_2HPO_4. To maintained 25% concentration, cooled 95% ethanol was added and maintained the temperature between -5°C and -6°C. Centrifugation removed the precipitated IgG and suspended in PBS [3]. Another method to obtain fractions from blood plasma is shown in Fig 2.

Electrophoretic methods: In electrophoresis, various particles bearing different charges are being separated. When the electric field applies across a solution containing differently charged particles. These particles move throughout the solution [4]. Different media are used for various types of electrophoretic enzymes, lipoproteins and serum proteins etc. Various media include polyacrylamide gel, cellulose and paper acetate, agarose gel and starch gel. Factors that affect the movement of proteins depend upon the shape and size of particles, medium temperature, viscosity and ionic strength of medium. Protein molecules move towards the anode due to negative charge at pH 8.6. Different fractions of the proteins like β and γ-globulin, albumin, α1, and α2-globulin are separated according to their charges. Electrophoretic methods include moving boundary electrophoresis, zonal electrophoresis, isotachophoresis, and SDS-PAGE. In moving boundary electrophoresis, fractions of proteins are separated by the boundaries of the medium. The apparatus includes buffer filled in a U- shaped cell and opposite electrodes are inserted into the medium. This electrophoresis cannot run at the intermediate range. It is run whether at pH 3.3 and 4.2 using citrate and acetate phosphate buffer or between pH 6.8 and 9.6 using borate-carbonate, phosphate-borate and phosphate buffers. The samples contained different proteins particles applied in electrophoresis. By applying voltage, positive and negative charges move towards their respective electrodes. Schlieren optics strips are used to detect particles at both ends by changing the refractive

index. Apparatus of moving boundary electrophoresis is shown in Fig (3) [5].

Zonal electrophoresis is improved than moving boundary electrophoresis. In which static medium is used for particle movement towards electrodes. Different proteins particles and serum samples move through a medium [6]. Zonal electrophoresis is used for the separation of M-proteins from serum through electrophoresis. In the following test, serum containing different immunoglobulins is mixed with cellulose acetate medium at basic pH. Proteins molecules present serum move towards anode by applying an electric field. After half an hour, the migration of particles is completed according to adjusted conditions. Cellulose acetate is dried dipped in Coomassie Blue, Ponceau SX stains and observed by naked eyes. The most visible and abundant protein present in serum is albumin. Sharp peaks showed serum proteins but in exceptional cases, albumin proteins showed the same electrophoretic results because the same amino acid sequences are present in most of the albumins, but gamma globulins have different amino acid sequences and different carbohydrate side chains. Ultimately, IgM and IgA move through medium towards β-globulins while IgG moves throughout the whole spectrum of globulins, but this method is not applicable for IgE and IgD. The electrophoretic pattern is changed by changing M-proteins producing plasma cells neoplasm [7].

Another electrophoretic technique is isotachophoresis (ITP) in which a boundary is created between particles within a sample by applying a discontinuous electric field to separate charged particles. In this technique, the proteins sample is poured between a fast-moving electrolyte and slow-moving electrolyte to establish a window to separate complex material from other constituents. An electric field is applied around electrolyte to separate charged particles. The electrolyte chose to run an experiment showed the excellent performance to remove contaminants from the final sample. The buffers used for the separation of ions must have good capacity in the adjusted pH range. When equilibrium is established, ions run at the same speed through an electrolyte. The movement of ions depends upon the spacer's mobility added to a solution containing a sample [8]. Isotachophoresis is useful for the purification of proteins but has a disadvantage that it can only separate positively and negatively charged ions through a capillary tube as have a property to move their respective electrode when an electric field is applied. Another method is capillary electrophoresis used for purification of specific proteins from proteins mixture. It is commonly used in proteins staking gel in which proteins samples are loaded within wells on a gel for separation of prominent bands based on molecular weight. Before the separation of proteins based on molecular weight, the proteins molecules are concentrated to increase resolution and obtaining small bands [9]. The purified serum proteins are used for the diagnosis of hemoglobinopathies, chronic liver disease, genetic abnormalities, malignancies, paraproteinemia's, immune deficiencies, and collagen disease. It also helps in the treatment of multiple myeloma [10].

The polyacrylamide gel electrophoresis (PAGE) using sodium dodecyl sulfate (SDS) was firstly performed in the 1960's [10]. Ionic surfactants are present in SDS for proteins denaturing. The proteins bind with surfactants by forming hydrophobic interactions and protein is folded by disturbing side-chain interactions. SDS-PAGE separates molecules based on molecular weight and size. Samples are treated on SDS and run on a gel. There is a difference in principle for various sample components based on molecular size and length. Larger molecules move slowly than smaller molecules. Time taken by molecules to run on polyacrylamide gel is inversely proportional to the molecular size of the sample. The sample is denatured and analyzed with a known molecular weight denatured sample to determine the analyte molecular weight. A hydrogen bond between proteins molecules is breakdown through urea. When urea is used as a denaturing agent without the presence of SDS, proteins molecules are not separated based on charge and size. If disulfide bonds are present in proteins molecules, mercaptoethanol (thiol reagent) is used to break the disulfide bond. Proteins do not contain a disulfide bond required 1.4 g of SDS for per gram of proteins comparatively 1g in per gram of non-reduced proteins. Finally, both non-reduced and reduced protein samples are run to determine internal disulfide bonds. Movement of bands on polyacrylamide gel are illustrated in Fig.4 [11].

Isoelectric focusing: Isoelectric focusing also called electro focusing to separate different ampholytes on the basis of their isoelectric points. The net charges of the proteins become zero at isoelectric point. A stable pH gradient is established by low molecular weight ampholytes. It is capable of detecting 100 mg of protein/L in the case of biological fluids in human using stains of conventional proteins. When other techniques such as probing and blotting techniques are combined together then its detecting capability to 1 mg/L. It shows greater differences in different immunoglobulin abnormalities performing in the laboratory [12].

Carrier ampholytes: Ampholytes are low molecular weight molecules such as proteins, amino acids, polyacid basic molecules. Ampholytes are used to generate a pH gradient in isoelectric focusing. Ampholytes having different isoelectric points are used in a mixture. In the case of proteins using as ampholytes, proteins continue to move through gel until pH becomes equal to their isoelectric point. If proteins are not used as ampholytes, then molecules other than proteins change their original pH. Ampholytes are mixed throughout gel and its cathodic and anodic ends are dipped into diluted base and acid respectively. A positive electrode, ampholytes will positively charge near the end of the gel while at the negative electrode, it will be negatively charged. Therefore, they start to move into gel until they separate into zones having different pH [13].

Principle: Isoelectric focusing runs in 2- dimensional electrophoresis. The tube gel system is included in apparatus. Glass tubes are used to caste the gel whose diameter is equal to the thickness of the second-dimensional gel. Gel of 1.5mm thickness is used. The gel is removed from the tube as the gel has run on IEF and placed on the second-dimensional gel. Slabs are also used to run the gel in IEF. A gel is cut after running on slab IEF to load on second-dimensional gels. The principle of isoelectric focusing is shown in Fig.5 [14].

Procedure: Gel preparation**:** The preparation of gel for protein analysis, 1mL of Proto Gel and 4g of urea dissolved

in 3 mL of water. Placed it at room temperature or warm at 37°C if necessary and use aspiration to remove gas for 10 minutes. 0.4 mL ampholyte and 150 µL Np-40 detergents are used and filtered by using 0.22 µm membrane filter. 3µL of TEMED and 30 µL of 10% ammonium persulfate are added. Mixed well and filled into a 10 mL syringe. A syringe is inserted into the gel tube, fill the tube and leave it for 2 hours for polymerization [15].

Sample preparation and running conditions: A buffer (15mL) containing (4% NP-40, 2% Mercaptoethanol having pH >9 with NaOH, 9M Urea and 2% Ampholytes having pH 9-11) is added into each gram of tissue. Mixed well and incubated for 10 minutes. Centrifuged for 1 hr at 100,000 g. The supernatant is removed and retained the pellet. Pellet is used to clog the IEF gel. 0.1% of phosphoric acid is used to fill the tank. The gel is placed in apparatus and 20mM NaOH is used to fill the upper tank. If bubbles have formed within the gel tube, remove them with the help of a syringe. Bubbles disturbed the electric field and prevent the focus. Fill the lower tank with 0.1% phosphoric acid. Place the gels in the apparatus and fill the upper tank with 20 mM NaOH. Use a syringe to dislodge any bubbles form inside the gel tubes. Any bubbles in the tubes will distort the electric field and prevent gels from completely focusing during the run. The prepared samples are loaded by forming layers on the top of the gel. Conditions to run the apparatus are changed for different apparatus but mostly 500-700V for 16-24 hours are required to run. In post electrophoresis, IEF gels are loaded on SDS PAGE for analysis of second dimensions. The pressure applied to one end of the tube by syringe or pipette while hold the other end of the tray to remove the gel. The gel is frozen at -70°C for later use [16]

Ion exchange chromatography: Ionex chromatography or ion-exchange chromatography is a technique that separates polar molecules and ions according to their affinity to the ion exchanger. The function starts on the base of charged molecules such as amino acid, proteins and small nucleotides [17]. Ion exchange chromatography has two types, cation exchange, and anion exchange.

Positively charged molecules are used in cation-exchange chromatography. The pH for the positively charged molecules is less than isoelectric point. In this chromatography, negatively charged molecules are present in the stationary phase and opposite charged molecules (negatively charged) are loaded for attraction. While in Anion-exchange chromatography, positively charged molecules are present in the stationary phase to attract negatively charged molecules. Movement of positive and negative charges towards their respective opposite charges is illustrated in Fig. (6) [18]. Ion exchange chromatography is performed for quality control, purification of proteins and water analysis. The charged and water-soluble molecules such as peptides, amino acids and proteins bind with the oppositely charged moieties to form ionic bonds [19].

Ion-exchange chromatography consists of negatively and positively charged ions and separation of molecules occurs according to their charged groups. It maintains analyte on column based on ionic interaction. On the stationary phase matrix, charged molecules interact with each other due to opposite charges. It contains the immoveable matrix for ligand bindings. Its surface exhibited functional groups to attract opposite charges. Exchangeable counterions attracted by active charged molecules to maintain electroneutral. For the purification of ionizable molecules, these molecules compete with exchangeable counterions for binding on stationary phase charges. These molecules are eluted according to their charges. Weakly bound molecules are firstly washed away from the stationary phase. It is needed to change the conditions by increasing the concentration of exchangeable counterions or changing the pH to elute the molecules from the stationary phase. The molecules are eluted out according to their charge adjustment. However, further modifications are needed to elute protein molecules. There are two types of elutions, one is gradient elution and the other is step elution. In gradient elution, a gradual increase of counterion concentration occurs to elute ionic molecules while in step elution, counterion concentration is the change in one step [17].

Before going to start ionic chromatography, it is necessary to equilibrate the stationary phase. The stationary phase contained charged ions to attract opposite ions. Exchange of ions occurs between charged ions and stationary phase ions. Mostly Na+ or Cl- ions are used in the stationary phase as exchangeable ions and for binding of proteins. The column is washed after equilibrium. All the impurities including uncharged proteins will wash out in the washing phase except bounded proteins. The buffer used for the binding of proteins has the same pH as the sample buffer. Salts concentration increased gradually for elution of bound proteins. Desired proteins compete with charged ions present on the surface by increasing the ionic strength of the buffer. Ultimately, desired proteins are eluted out. The apparatus and principle of ion-exchange chromatography is illustrated in Fig: (7)

In chromatography column, counterions such as copper (II) are applied for gradually increasing gradient in a column. It separates peptides and amino acids to form a complex [20]. Individual proteins can also be eluted according to their isoelectric point by applying a pH gradient. The isoelectric point at which amino acids molecules in a protein do not move in an electric field by adopting a neutral charge. Proteins molecules contain all three types of charges (positive, negative and neutral) due to the presence of zwitterionic compounds. Proteins molecules are eluted without interacting with column resins containing charge moieties. In anion exchange resins, it is necessary to decrease the pH to elute proteins while in cation exchange resins pH gradient is increased to elute proteins. Because the pH of the buffer used in the mobile phase allows the proteins to reduce positively charge so that it becomes unable to interact with negatively charged resins, proteins are eluted. Besides this, proteins molecules are eluted by reducing the pH of the mobile phase because they are more protonated having a less negative charge. Stationary phase contains immobilized charged groups such as diethyl-2-hydroxypropylaminoethyl (QAE), carboxymethyl (CM), aminoethyl (AE), sulfur (S), trimethylaminoethyl (TAM), sulphopropyl (SP), triethylaminoethyl (TEAE), sulphomethyl (SM) and carboxy (C) [17].

There are different mediums used for separation of different sized proteins such as polystyrene is made by combining benzoyl peroxide and divinylbenzene.

Polystyrene is used for the removal of small molecules like amino acid and salts from water. It cannot remove large molecules like proteins due to its ability to form irreversible hydrophobic interactions with proteins. Protein molecules are also separated using large polystyrene ion exchangers containing. Such polystyrene must be coated with hydrophobic substances [21]. Large molecules like proteins are also separated by cellulose-based medium can be used for the separation of large molecules as they contain large pores. The efficiency of ionic chromatography is increased by increasing temperature, pressure and decreasing time. Adjustment of temperature depends on retention properties. Retention factor decreased for large ions and it is opposite for small ions [22,23]. Instead of selecting different mediums, the research is being carried out to perform ion-exchange chromatography at the range of 40–175 ^{o}C [24].

Immunoglobulins from serum blood are also isolated from ion-exchange chromatography. Different columns like QAE-Sephadex, DEAE-Sephadex DEAE-cellulose, and QAE-Sephadex are used for the purification of IgG. A purified IgG is obtained in a single separation through a chromatography column. Ion exchanger is often packed in column and DEAE-Sephadex is mixed with buffer containing 0.015 mmol/L phosphate with pH 6.5 and serum in a beaker. After a long time mixing, filtered the mixture to obtain purified IgG. IgG binds weakly with anion exchanger than IgM. IgM is mixed with other proteins present in serum therefore, the highly ionic solution is required for elution hence IgM is not purified by ion-exchange chromatography [25].

Gel filtration chromatography: In this technique, only proteins molecules of various molecular sizes are separated. A porous matrix is used for the separation of molecules in which smaller molecules have a greater ability to pass out than larger molecules. In the chromatographic column, matrix and aqueous buffer are used in which aqueous buffer is acts as a mobile phase for the separation of molecules. Molecules of Protein are present outside the matrix beads. Proteins molecules are eluted according to their sizes through mobile phases with the column. UV monitor is used to detecting and fractionation of eluted proteins for further analysis. Different adsorptive techniques are selected for further purification and polishing. In the polishing stage, properties of molecules similar to target proteins are removed. Various natural and synthetic compounds are used in a column. Proteins and enzymes (purified the fish) having molecular sizes $4.3 \times 10^{4,}$ 1.19×10^{5} are separated by Superdex 200 compounds respectively [26]. In industries and laboratories, high-quality separation is achieved by Sephacryl and Superdex prep grade [27,28]. Trypsin inhibitors isolated from Yellowfin Tuna *(Thunnus Albacores)* roe are purified by chromatography column containing DEAE-cellulose, Sephacry S-200, and Sephadex G-50. Besides this, Sephadex is used for the separation of buffer exchange and desalting. This column is widely used for marine organisms. Sephadex G-75 purifies the marine yeast while Sephadex G-25 to mussel flavor and *Parastromateus Niger*.

Method: Gel filtration chromatography is used for proteins and polymers separation. The process is carried out in a column containing porous beads of different sizes. Smaller molecules are trapped in the adsorbent present in the stationary phase. The pores in beads provide channels for the passing of molecules. when solution moves through the column, smaller particles trapped in pores while larger molecules are eluted. Larger molecules move more quickly than smaller molecules due to increase retention time. Analyte mustn't touch the surface of the stationary phase. The elution of analyte is based on the volume of the solute instead of electrostatic and chemical interactions. The column's 80% volume relies on the stationary phase in which interparticle and total pores volume is included. However, there is a problem in GF to select parameters to measure the proper size of the molecules to separate. Benoit and fellows discovered a good relationship between the size of the molecules and elution volume. The principle of gel filtration chromatography is shown in Fig (8) This relationship is established based on hydrodynamic volume. But to interpret GF data is not fully understood by hydrodynamic volume [29]. In GF, hydrodynamic factors do not affect the separation of molecules by following low flow rate conditions. The separation process is based on establishing equilibrium between two phases; confined solution phases established in matrix filled within a column and dilute bulk solution phases present in interstitial space [30].

For the separation of immunoglobulin, Sephadex G-200 column is filled by a mixture containing different immunoglobulins, IgM is firstly eluted from a matrix. Different ingredients such as 0.5mol/L NaCI in 0.02 mol/L phosphate buffer, pH 7.3 and high salt concentration are used in buffer. Ultimately, human serum is separated into three peaks. In the first peak, lipoproteins and macroglobulin are eluted but IgA is separated in the last portion of a fraction. Agarose gel electrophoresis is used for the separation of macroglobulin and IgM. In which macroglobulin migrates to the anode while IgM to the cathode. In the second peak, the largest part of IgG is separated from IgA. In the third peak, other proteins present in serum and albumin proteins are separated. Separation of the first two peaks is not well satisfied because polymers and dimers formed by aggregation of IgG molecules. This mixing of IgG blocks the separation of IgM from IgG (2).

Hydrophobic chromatography: Proteins are purified by developing an association between a matrix in chromatographic columns and proteins. The matrix material present in the chromatography column is divided into groups such as methyl, ethyl, propyl, octyl, or phenyl groups (17). When the concentration of salts is increased, hydrophobic groups are interacted by the non-polar side chain of proteins. Ionic strength depends on hydrophobic effects. However, polar solvents removed both types of groups. The column containing buffer is suitable for high polarity samples. The buffer contained eluent is eluted by increasing detergent concentrations, changing pH and decreasing salt concentrations.

Hydrophobic chromatography is beneficial for highly sensitive samples to pH and solvents but not the presence of a high concentration of salts because its concentrations change in a buffer. Bovine Serum Albumin (BSA) and four other types of resins are used to check the effects of temperature on chromatography. The binding affinity of the BSA to the surface of the matrix is changed by

changing temperature. Finally, the conducted study concluded that decreasing temperature from 5°C to 1°C does not wash all BSA from the column. Hydrophobic adsorbents are prepared by the agarose matrix. Mostly Phenyl-Sepharose and OctylSepharose are used in matrix adsorbents. The non-polar radicles such as phenyl or octyl groups formed hydrophobic bonds. The separation process of hydrophobic chromatography is similar to affinity chromatography. In the hydrophobic column, a sample is mixed in a solution with a pH approximately equal to the isoelectric point of the protein. Desired proteins are bounded with the matrix in a column while other contaminants are eluted out. These bounded proteins are eluted by reducing hydrophobic interactions, decreasing polarity and ionic strength of the eluent. In the case of immunoglobulins, only the IgG antibody is isolated. Phenyl-Sepharose CL 4B column is filled with 1 mol\L of $(NH4)_2S0_4$ in which the IgA antibody is bounded while others are eluted. Then bounded IgG antibody is eluted by using 0.8 mol\L $(NH4)_2S0_4$. Single gel filtration step is used for the re-purification of the IgG antibody [31].

Conclusion: All these artificial techniques are very important for the bulk production of immunoglobulins Because they are very essential for the diagnosis of different diseases like ELISA based diagnosis in a purified and mainly in labelled form. We can also assume that they are even very important in RIA (Radio immuno assay's) based cancer diagnosis. These are more reliable techniques as compared to the extraction of immunoglobulins from the animals.

References

1. Sun, Yuanjie, et al. "Preparation of antibodies against TXR1 and construction of a new DNA tumor vaccine." *International Immunopharmacology* 103 (2022): 108505.
2. Ferencik M. Handbook of immunochemistry. Springer Science & Business Media; 2012.
3. Sun, Haofeng, et al. "Epitope mapping of antibodies in C-reactive protein assay kits by hydrogen-deuterium exchange mass spectrometry explains differential results across kits." *Analytical and Bioanalytical Chemistry* 414.13 (2022): 3875-3884.
4. Rathore, Anurag S., and Sharad Narnaware. "Purification of therapeutic antibodies by protein a affinity chromatography." *Therapeutic Antibodies*. Humana, New York, NY, 2022. 169-177.
5. Righetti PG %J J of CA. Electrophoresis: the march of pennies, the march of dimes. 2005;1079(1–2):24–40.
6. Guzman NA, Park SS, Schaufelberger D, Hernandez L, Paez X, Rada P, et al. New approaches in clinical chemistry: on-line analyte concentration and microreaction capillary electrophoresis for the determination of drugs, metabolic intermediates, and biopolymers in biological fluids. 1997;697(1–2):37–66.
7. Baird St. Louis, San Francisco SM %J WH 6th ed. MHNY. Plasma cell neoplasms: general considerations. 2001;1263–70.
8. Inano K, Tezuka S, Miida T, Okada M %J A of clinical biochemistry. Capillary isotachophoretic analysis of serum lipoproteins using a carrier ampholyte as spacer ion. 2000;37(5):708–16.
9. Young CC. Purification and concentration of proteins and DNA from a complex sample using isotachophoresis and a device to perform the purification. Google Patents; 2013.
10. Whicher JT, Calvin J, Riciies P, Warren C %J A of clinical biochemistry. The laboratory investigation of paraproteinaemia. 1987;24(2):119–32.
11. Rudge SR, Monnig CA. Electrophoresis Techniques. Sep Purif Methods. 2011;29(1):129–48.
12. Cornell FN %J TCBR. Isoelectric focusing, blotting and probing methods for detection and identification of monoclonal proteins. 2009;30(3):123.
13. Sebastiano R, Simó C, Mendieta ME, Antonioli P, Citterio A, Cifuentes A, et al. Mass distribution and focusing properties of carrier ampholytes for isoelectric focusing: I. Novel and unexpected results. 2006;27(20):3919–34.
14. Friedman DB, Hoving S, Westermeier R. Isoelectric focusing and two-dimensional gel electrophoresis. In: Methods in enzymology. Elsevier; 2009. p. 515–40.
15. Gallagher SR, Desjardins P %J CPELT. Quantitation of nucleic acids and proteins. 2011;5(1):2.2. 1-2.2. 36.
16. Mostovenko E, Hassan C, Rattke J, Deelder AM, van Veelen PA, Palmblad M %J EOP. Comparison of peptide and protein fractionation methods in proteomics. 2013;1:30–7.
17. Ninfa AJ, Ballou DP, Benore M. Fundamental laboratory approaches for biochemistry and biotechnology. John Wiley & Sons; 2009.
18. Dąbrowski A, Hubicki Z, Podkościelny P, Robens E %J C. Selective removal of the heavy metal ions from waters and industrial wastewaters by ion-exchange method. 2004;56(2):91–106.
19. Luqman M. Ion exchange technology II: applications. Vol. 2. Springer Science & Business Media; 2012.
20. Dieter DS, Walton HF %J AC. Counterion effects in ion-exchange partition chromatography. 1983;55(13):2109–12.
21. Janson J-C. Protein purification: principles, high resolution methods, and applications. Vol. 151. John Wiley & Sons; 2012.
22. Wouters B, Bruggink C, Desmet G, Agroskin Y, Pohl CA, Eeltink S %J A chemistry. Capillary ion chromatography at high pressure and temperature. 2012;84(16):7212–7.
23. Escuder-Gilabert L, Bermúdez-Saldaña JM, Villanueva-Camañas RM, Medina-Hernández MJ, Sagrado S %J J of CA. Reliability of the retention factor estimations in liquid chromatography. 2004;1033(2):247–55.
24. Shibukawa M, Shimasaki T, Saito S, Yarita T %J A chemistry. Superheated water ion-exchange chromatography: an experimental approach for interpretation of separation selectivity in ion-exchange processes. 2009;81(19):8025–32.
25. Tijssen P %J L techniques in biochemistry, Practice molecular biology., theory of enzyme immunoassays London, New York, Tokyo, Elsevier A. Kinetics and nature of antibody-antigen interactions. 1993;123–49.
26. Qian L, Zhang Y, Liu F %J J of the S of F, Agriculture. Purification and characterization of a∼ 43 kDa antioxidant protein with antitumor activity from Pholiota nameko. 2016;96(3):1044–52.
27. Iberer G, Schwinn H, Josić D, Jungbauer A, Buchacher A %J J of CA. Improved performance of protein separation by continuous annular chromatography in the size-exclusion mode. 2001;921(1):15–24.
28. Shen J-D, Cai Q-F, Yan L-J, Du C-H, Liu G-M, Su W-J, et al. Cathepsin L is an immune-related protein in Pacific abalone (Haliotis discus hannai)–Purification and characterization. 2015;47(2):986–95.
29. Sun T, Chance RR, Graessley WW, Lohse DJ %J M. A study of the separation principle in size exclusion chromatography. 2004;37(11):4304–12.
30. Wang H, Lee E-W, Cai X, Ni Z, Zhou L, Mao Q %J B. Membrane topology of the human breast cancer resistance protein (BCRP/ABCG2) determined by epitope insertion and immunofluorescence. 2008;47(52):13778–87.
31. Müller TKH, Franzreb M %J J of CA. Suitability of commercial hydrophobic interaction sorbents for temperature-controlled protein liquid chromatography under low salt conditions. 2012;1260:88–96.

.

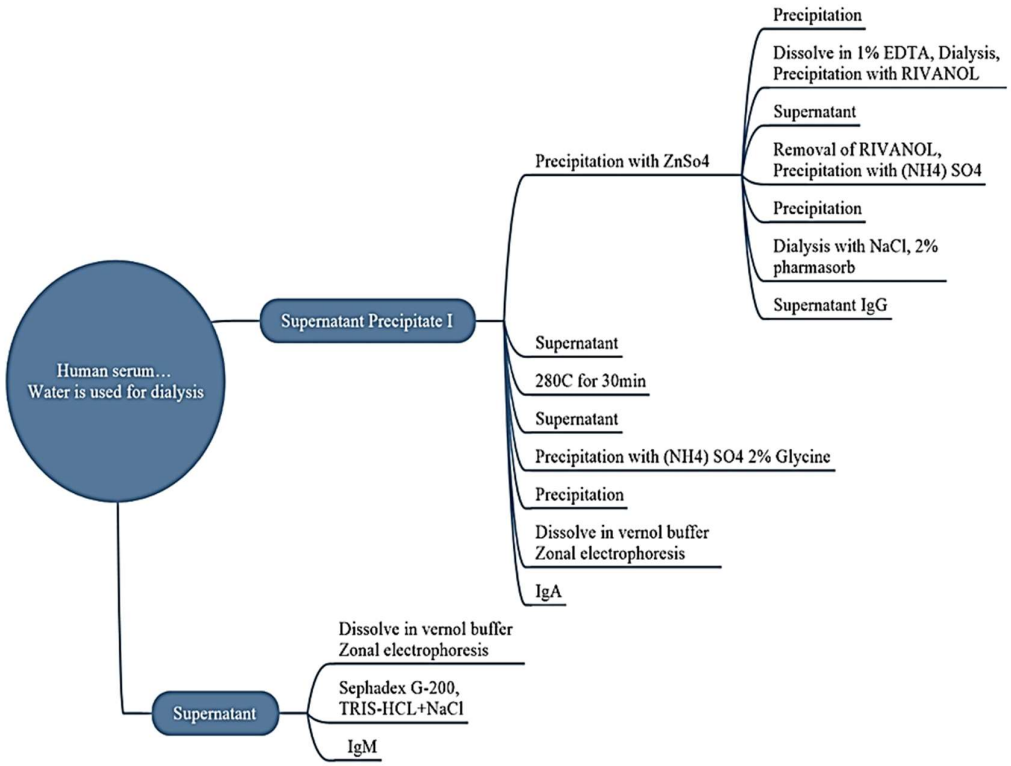

Fig. 1. A method for isolation of IgA, IgG, IgM from serum sample

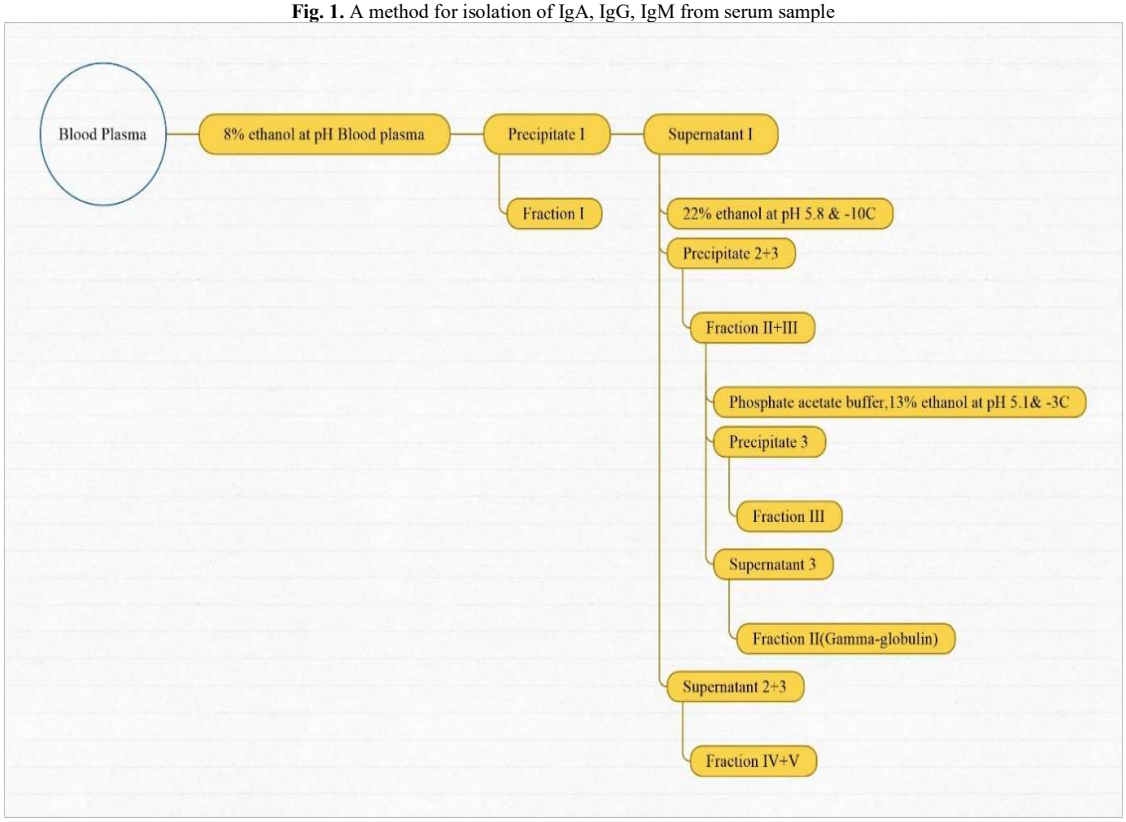

Fig. 2. Blood plasma fractionation using ethanol.

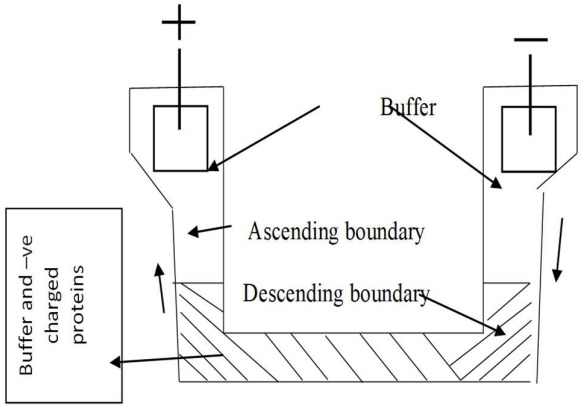

Fig. 3. Apparatus of moving boundary electrophoresis

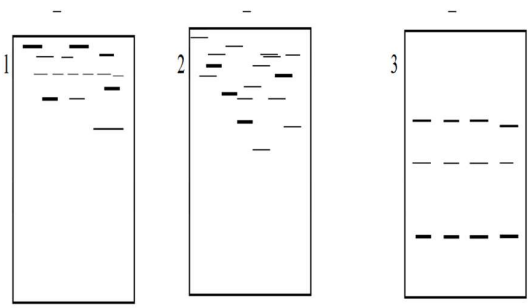

Fig. 4. Principle of SDS-PAGE, proteins with higher molecular weight move faster on gel than low molecular weight proteins

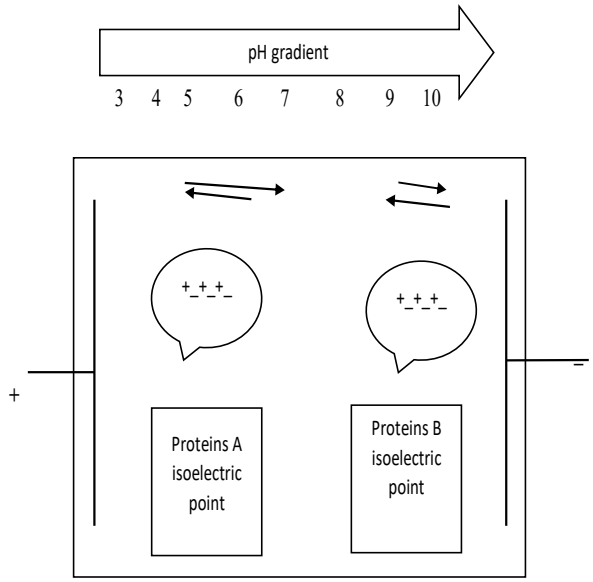

Fig. 5. Principle of isoelectric focusing of two different proteins with different isoelectric points

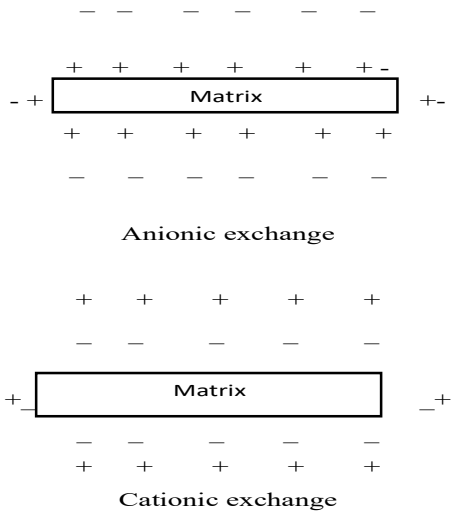

Fig. 6. Principle of ion exchanger

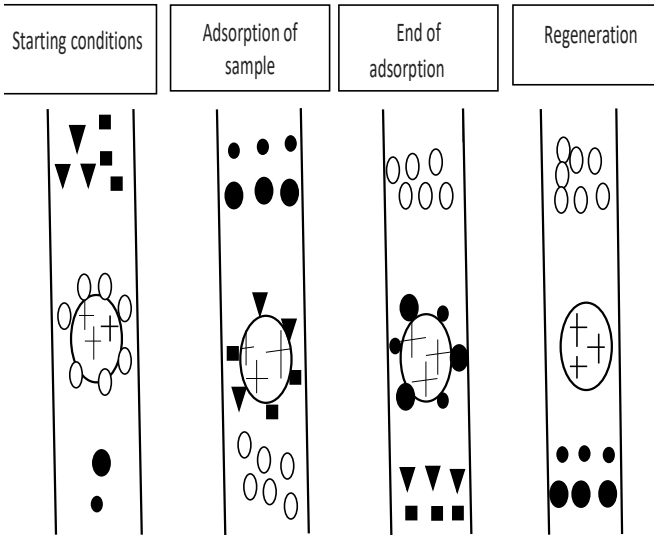

Fig: 7 Principle of ion-exchange chromatography starting buffer encounter with + ions, substance to be separated Gradient ions.

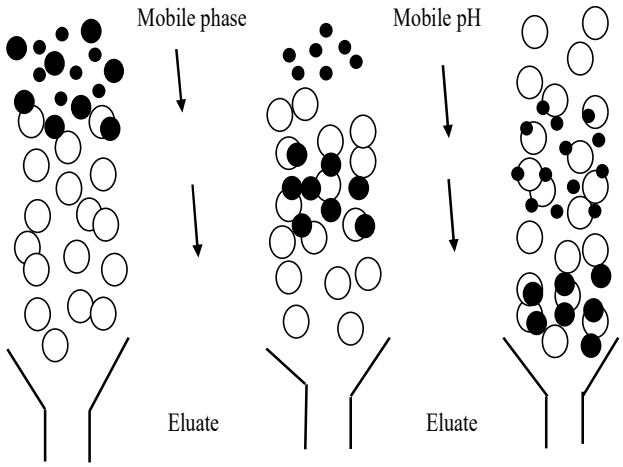

Fig: 8 Principle of gel filtration electrophoresis