OCT 2022
ISSUE 10

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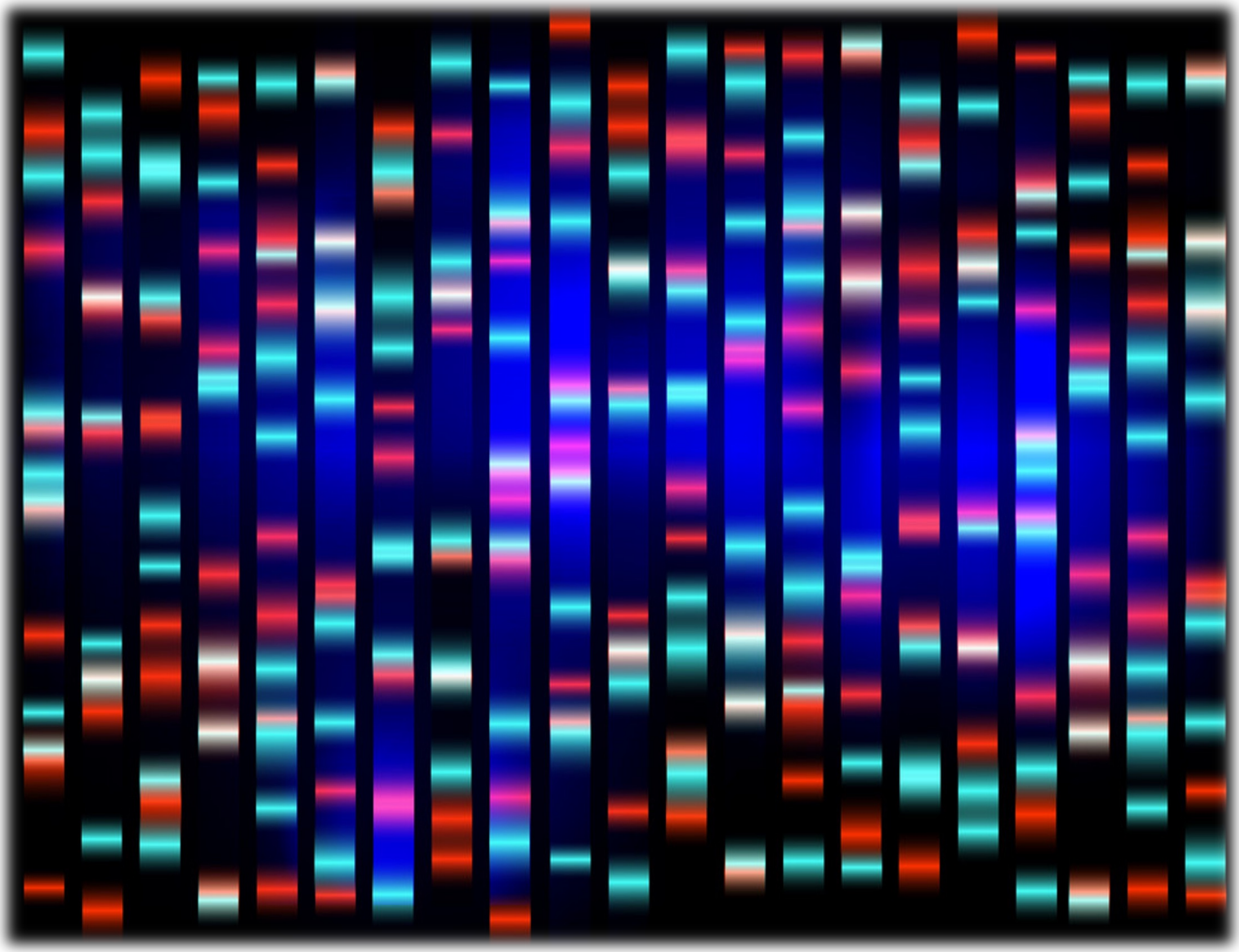
News bulletin of AMBI West Bengal Chapter

The Biochemistry Chronicles



Serum Protein Electrophoresis : A boon in Disguise for clinicians

Introduction :

Serum protein electrophoresis is an underused laboratory technique of separating proteins present in the serum to various fractions based on their molecular weight and electric charges. It's quite an inexpensive screening technique which in clinical practice is limited to the diagnosis of patients with multiple myeloma and other serum protein disorders. However, Serum protein electrophoretogram if studied minutely, it may give insight to various underlying pathologies, common diseases with unusual presentations and rare disorders with typical presentations in many patients. Thus, serum protein electrophoresis needs to be popularized amongst physicians and hence learning to interpret the electrophoretogram is altogether important for the Biochemist for an effortless error free interpretation of the report.

Technique basics:

We all have been accustomed to indigenous gel electrophoresis techniques in our post graduate labs, however while reporting for patients in private hospitals / standalone labs we prefer the use of commercially favourable instruments for a better throughput.

The separation of proteins by electrophoresis is based on the fact that charged molecules usually migrate through a matrix/medium on application of an electrical field. The rate at which proteins move in an electric field is determined by a number of factors of the electrophoretic system like the strength of the electric field, temperature of the system, pH of the ions, concentration of buffer etc. & also the nature of the proteins. Proteins vary in their size and shape and have the charges determined by the their amino acids content. Smaller proteins migrate faster usually while larger proteins take a longer time. This physical property of proteins is exploited for its separation by employing the electrophoretic technique. The most commonly employed variant of electrophoresis for serum protein separation is zone electrophoresis in which the serum proteins are separated into zones or fractions and interpreted accordingly. There are several support mediums available for separation of serum proteins including agarose, cellulose acetate, capillary medium etc. Capillary electrophoresis is the preferred method when compared to its competitors including agarose gel electrophoresis due to the following reasons. It provides an improved resolution due to the following factors:

a. The use of "electroendosmosis" principle which improves the resolution of separation

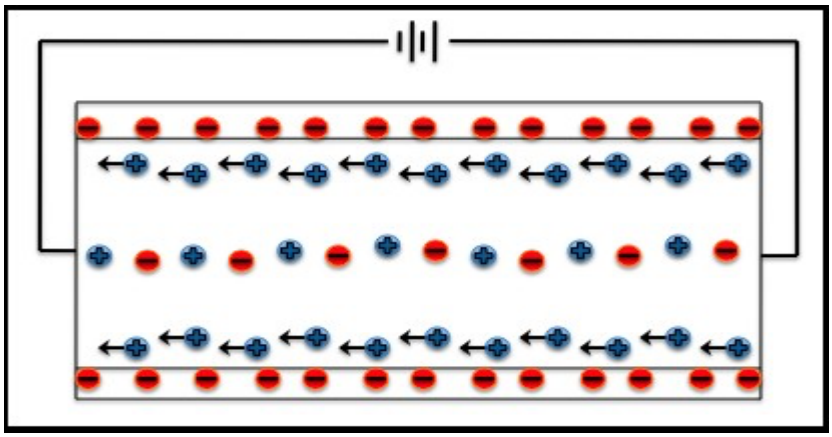


Figure 1: Pictorial representation of electro-osmotic flow

The electro-osmotic flow (EOF) is generated when electrodes are placed in the reservoirs at each end of a microchannel by a process known as electro-osmosis. The EOF is implemented through the surface charges dominant in the small scales. The surfaces of most channel materials (e.g., glass and polymer) are negatively charged in an electrolyte solution. This causes a surplus of positively charged anions in the double layer or Debye layer close to the channel walls. Under an electric potential along the channel, the excess charges in the double layer are attracted by electrostatic forces, and thus, move toward the negative electrode. Because of the viscous coupling, the bulk liquid is pumped by the mobile layer, and its original flat shape is maintained with less sample dispersion within an EOF. (1)

b. Employing a "high-voltage" electric curr -ent which aids in improving the throug -hput (the processing time) and the resolu-tion of protein separa -tion.

Newer systems use the principle of capillary electrophoresis (CE) in free solution. Charged molecules are separated by their electrophoretic mobility at a specific pH in an alkaline buffer. Separation occurs according to the electrolyte pH and electroosmotic flow. CE has a steady flow, compared to the pumped parabolic flow of the High-Performance Liquid Chromatography technique (HPLC). The steady flow results in narrower peaks and better resolution. The latest capillary electrophoresis instruments are equipped with several parallel capillaries enabling multiple simultaneous analyses and scalable throughput.

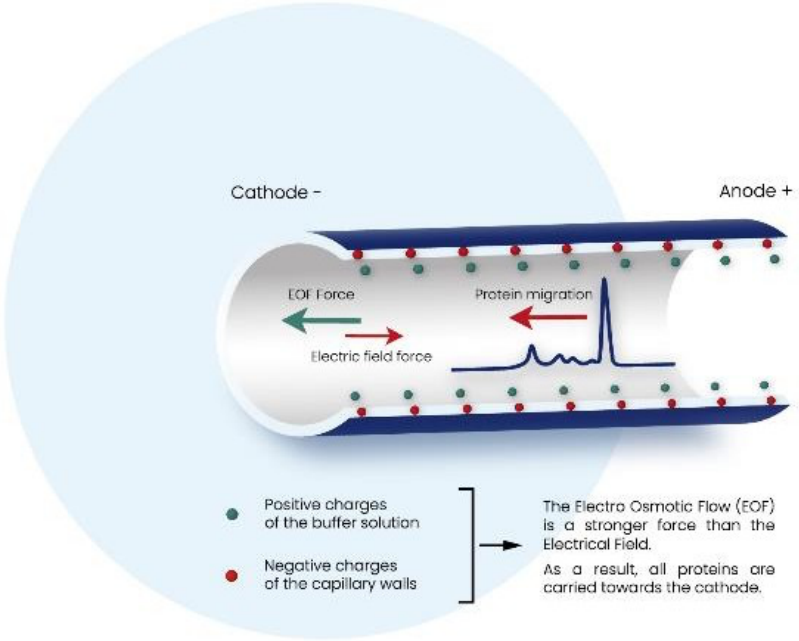


Figure 2: Diagrammatic representation of the principle of Capillary electrophoresis

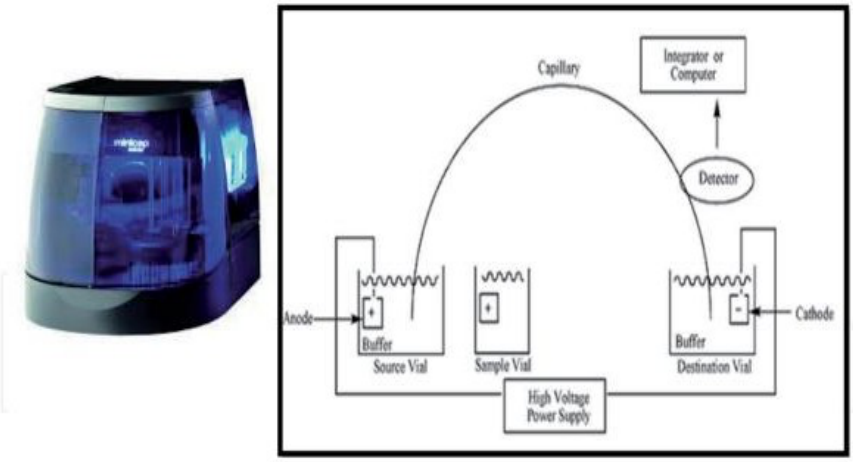


Figure 3: A capillary electrophoresis system-(Sebia Minicap flex piercing capillary electrophoresis)

The above figure (FIG:3) shows a capillary electrophoresis system which aids in testing of human blood with capped tubes which in turn eliminates the biohazard associated with handling of uncapped samples.

Quality control:

Like all other test parameters / methods, quality assurance is a prerequisite for ensuring reliability of an SPE result. Major aspects of analytical quality include precision (measure of repeatability) and accuracy (measure of trueness or reliability).

Good clinical laboratory practices demand processing of an internal quality control (IQC) for assessment of precision and external quality assurance (EQA)/ proficiency testing (PT testing) for accuracy assessment. IQC material can be prepared in house (patient sample) or availed commercially and run before patient sample processing. The clinical laboratory should select and use an IQC which has a matrix comparable to patient sample, preferably covering the clinical decision point (cut off value that differentiates between a normal and abnormal result). EQA is an external assessment of the analytical quality where the laboratory processes a blinded sample and the results are compared against a referencemethod and/or against the consensus value of other participant laboratories for that specific sample (peer group). The EQA sample provider shall preferably be accredited to ISO 17043 whenever not possible the lab should undergo Interlab comparison with labs using similar instrument / procedure and also to take care of the accreditation status of the comparing lab for the test.

For daily reporting of SPE by one of the latest Capillary Electrophoresis system, most of the labs use commercially available controls, one normal and one hypergamma before processing patient sample for the day.

Components of Serum Protein Electrophoresis:

Two major types of proteins can be identified with SPE: Albumin and Globulins. Albumin is the major fraction synthesized endogenously in liver and exogenously available through various dietary sources including egg, meat, pulses, milk etc. Globulins are a group of proteins sub - classified into alpha-1, alpha-2, beta-1, beta-2, and gamma globulins based on their electrophoretic mobility. The normal biological interval of serum total protein in a healthy adult range between 6 and 8 g/dl, Serum Albumin: 3.5–4.5 g/dl and Globulins: 2.5–3.5 g/dl.

Table 1: Electrophoretic distribution of serum protein

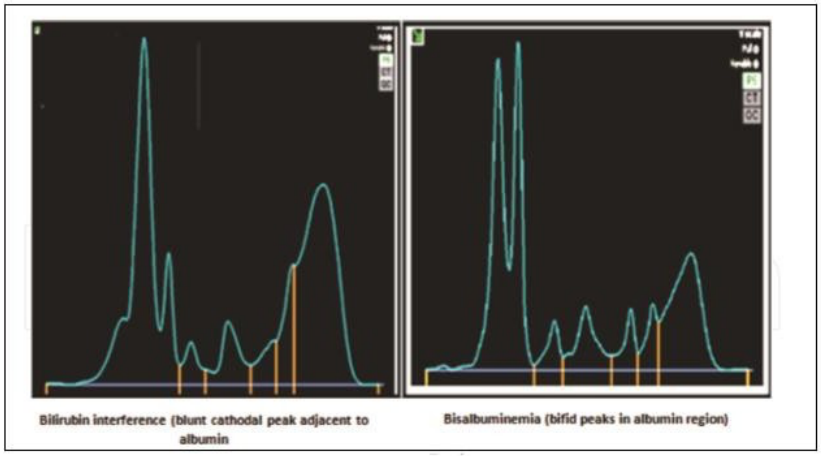
	Distribution %	Absolute amount, g/l
Albumin	55	40
α_1-Globulin	5	4
α_2-Globulin	10	7
β-Globulin	12	9
γ-Globulin	18	13
Total	100	73

1. **Albumin**: Albumin is a 69 kDa protein consisting of 585 amino acids which are organized into three repeated homologous domains and are made up of two separate sub-domains, A and B. It is the most abundant protein in serum. It is produced by the liver hepatocytes under normal physiologic conditions rapidly excreted into the bloodstream at the rate of about 10 gm to 15 gmper day. Human albumin acts as the most significant modulator of plasma oncotic pressure and functions to transport a variety ligand, endogenous ligands such as bilirubin, ions, fatty acids, and exogenous ligands such as drugs, methadone, propranolol, thiopental, furosemide, warfarin,methotrexate, alfentanil, to name a few. On the graphical output of SPE

it forms the largest peak and lies closest to the positive electrode (anode) on the left side of the panel. A fall in serum levels of larger than 30% is necessary to be detected with electrophoresis. Decreased concentration of albumin (Hypoalbuminemia) may occur in poor dietary intake (Malnutrition), decreased albumin production (liver failure), increased loss of albumin in urine (Nephrotic syndrome), and also in acute and chronic inflammation, hormone therapy, burns or pregnancy.

Apart from hypoalbuminem -ia, a few more variations may occur in albumin peak in electrophoretic run:

a. Occasionally, a single wide band or two bands of unequal intensity may also be seen, but both of these variants are not associated with any disease. The formation of these variants results from increased mobility of albumin due to binding of bilirubin, nonesterified fatty acids, penicillin or acetylsalicylic acid to albumin.
b. Prealbumin (transthyretin)— increased levels of pre albumin, if present due to various clinical conditi- ons including several inflammatory diseases is seen as a blunt anodal peak distinctly separated from the peak of albumin.
c. Bisalbuminemia is a rare electrophoretogram finding characterized by bifid albumin peak in densitometric scan. The incidence of these variants is usually stated to be 1:1000 to 1:3000. Bisalbuminemia may be inherited or acquired in nature. When inherited it is of autosomal dominant (occurring with a cumulative frequency of 1:1000-1:10,000) in nature, however acquired bisalbuminemia which is mostly an incidental finding, can be seen in patients with pancreatic pseudocyst rupture, Diabetes mellitus, Cirrhosis, hyperamylasemia, nephrotic syndrome, chronic kidney disease, sarcoidosis, Alzheimer's disease, plasma cell dyscrasia like Waldenstrom's macroglobulinemia and patients taking beta lactum antibiotics.
d. Analbuminemia (absence of albumin) is another genetically inherited metabolic disorder and was first described in 1954. This disorder is rare and affects less than 1 in 1 million births.



2.The Globulins: The globulins comprise of a much smaller fraction of the total serum protein content. The four components of globulins are labeled α 1, α 2, β and γ. The peaks for these components lie towards the negative electrode (cathode) on the right side of the graphical output, with the γ -peak being closest to the cathode.

Albumin– α 1- Interzone: Even staining in this zone is due to α 1 -lipoprotein (high-density lipoprotein). A decrease can be found in severe inflammation, acute hepatitis and liver cirrhosis or in nephrotic syndrome. An increase of the α 1 -interzone can be found in severe alcoholism and in physiological circumstances such as in women during pregnancy or in young people during puberty. High levels of α-fetoprotein (AFP) from hepatocellular carcinoma may also result in a sharp band in the

α 1- interzone. ***α 1 -Zone*** The α 1 -protein fraction is comprised of AFP, α 1-glycoprotein, thyroid-binding globulin, and transcortin. A decreased band is seen in deficiency states like AFP deficiency, nephrotic syndrome and liver failure from cirrhosis. Bence Jones protein from multiple myeloma however may bind to and retard the α 1- band and thus causing a decreased α 1- zone, where -as in other malignancies or during inflammatory responses there may be an increase of the α 1- protein band from acute-phase reactants.

α 1–α2 – Interzone: Two faint bands may be seen representing α 1 – anti -chymotrypsin and vita -min D binding protein. These bands, however, fuse and intensify in early inflamm -ation due to an increase in α 1 – antichymotrypsin which is an acute-phase protein.

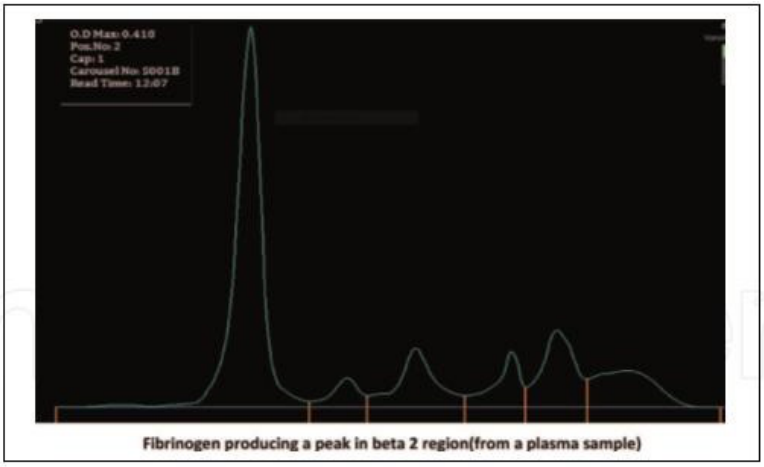
α2 –Zone: Ceruloplasmin, α 2- macroglobulin, and haptoglobin contribute to the α 2- protein band. The α 2-zone is typically decreased in hemolytic anemia when haptoglobin binds with free hemoglobin from red blood cells and these complexes are rapidly removed by phagocytes. Haptoglobin being a acute phase protein may also be elevated, especially during inflammation. Children and elderly people show higher levels of α 2 -macroglobulin and may present a sharp front to the α 2- band. In nephrotic syndrome the α 2- zone may be increased relative to other protein bands because of their inability to pass through glomeruli (due to their size). The α2 -band may be raised in liver cirrhosis, diabetes mellitus and malignancies (acute-phase reaction). Ceruloplasmin is an important copper-binding transport protein produced by the liver. Ceruloplasmin concentrations are markedly decreased in conditions of Wilson's disease. However, the disadvantage of serum protein electrophoresis is that it will not aid in the detection of a decreased ceruloplasmin.

β- Fraction: The β- fraction may be separated into a β 1- band and a β 2- band but on graphical data is often represented as a single band. Transferrin & low-density lipoprotein (LDL) comprises the β 1 -band. An increase in of β 1- proteins is typical for iron-deficiency anemia due to elevated levels of free transferring, pregnancy and estrogen therapy. Determinations of the transferring levels are useful in distinguishing between iron deficiency anemia (inadequate intake or chronic hemorrhage with loss of iron stores) and hemolytic anemia, in which transferrin levels are low resulting in a low beta-1 peak.

Transferrin, however, is usually decreased in alcoholic cirrhosis, in renal disease and thermal injuries.

The β 2- band is formed by complement protein 3 (C3) and β -lipoprotein. IgA (immunoglobulin A), IgM, and sometimes IgG also can be identified in the β- fraction. Elevated β-2 zone can be seen in inflammatory states due to activation of complement cascade which include both C3 and C4 . A reduced beta-2 peak intensity can be found in an aged sample, since the immune complexes are used up and insuch cases it is evidenced by low serum levels of complements.

Fibrinogen is a protein with molecular weight of 340 kDa protein. Sometimes a small fibrinogen band can be seen in serum protein electrophoresis due to the insufficient clotting or failure to remove the serum from the clot. This fibrinogen band is seen between beta-1 and beta-2 regions. This band is also seen in patients who are receiving heparin therapy. It is also an important indicator of the sample type being analyzed. When plasma is used in the place of serum for protein electrophoresis, fibrinogen present in plasma appears in the beta-2 region, and this is a potential interference to the detection of monoclonal gammopathies in such patients. Thus bands in beta 1 and beta 2 bands should be reported with care.



Fibrinogen producing a peak in beta 2 region(from a plasma sample)

Gamma fraction : The main clinical implication of SPE is diagnosis of disorders associated with with alterations of gamma globulins. The immunoglobulins are characterized by the presence of two protein moieties named as heavy chain and light chain. The classification of immunoglobulin had been made based on the composition of heavy chains, while the light chains are of two types including kappa or lambda. Physiologically, kappa forms the major proportion of the light chain fraction amongst the two. The various immunoglobulin classes (IgG, IgA, IgM, IgD and IgE) are usually of γ -mobility and make up most of the γ- band, but they can also be found in the β –γ- and β- regions, and may occasionally even extend into the α 2- globulin area.

Conditions associated with alteration in gamma globulin region:

a. Hypogammaglobinemia (decreased serum gamma globulin levels)

b. Hypergammaglobinemia (increased serum gamma globulin levels)

- The γ-globulin zone is decreased in agamma-globulinemia and hypogammaglobulinemia syndromes. Agammaglobulinemia is comprised of the following types:

a. X- Linked Agammaglobulinemia
b. X - Linked Agammaglobulinemia with Growth Hormone Deficiency
c. Autosomal Recessive Agammaglobulinemia (ARAG)

Hypogammaglobinemia may be found in newborns also. IgA deficiency occurs in 1 out of 500 patients and often remains undetected. On SPE, IgA has the most anodal mobility (closer to the left side of the graphical output) and may lead to pallor in the γ-zone.

Hypergammaglobulinemia: Gammopathy is defined as abnormal proliferation of the lymphoid cells producing immunoglobulins. Gammopathies can be classified as: polyclonal, monoclonal, biclonal, and oligoclonal.

Polyclonal gammopathies are defined as heterogene -ous increase in immunoglo -bulins involving more than one cell line whereas Monoclonal gammopathies are characterized by a homogenous increase in immunoglobulins, produced by clonal population of mature B cells, most commonly plasma cells.

Monoclonal immunoglobul -ins known as Para proteins are classically interpreted in SPE as “M” band where M stands for monoclonal. Usually 60% of cases of multiple myeloma & plasmacytoma present with “M” Band in SPE while approximately 10% of cases of Waldenströms Macroglo -bulinemia, lymphomas, and leukemia do so. Certain monoclonal gammopathies also produce “M” band in electrophoretic regions other than in gamma region in beta regoin especially in

beta region especially in case of IgA and IgG myeloma. Biclonal gammopathies are characterized by a double peak in the gamma region.
This electrophoretic pattern is seen when there is a biclonal proliferation of immunoglobulins encountered in multiple myeloma. A biclonal pattern is also seen in monoclonal gammopathies associated with IgA and IgG. In such scenarios, these immunoglobulins appear as polymerized and monomerized forms which elute as biclonal peaks in gamma region or in beta region, respectively. These are more clarified using Immunotyping or Immunofixation. The oligoclonal pattern of gamma region is characterized by more than two peaks evident in the gamma region. This pattern is commonly seen in autoimmune disorders, light chain myelomas (characterized by clonal proliferation of light chains), amyloidosis, etc. Apart from serum immunoglobulin, C-reactive protein (CRP) also is evident in the gamma region. C-reactive proteins levels usually increase during inflammatory responses.

Apart from the common causes of altered electrophoresis picture specific to the particular zones, a sharp distinct peak when evident especially in beta or alpha region should raise a high index of diagnostic suspicion of multiple myeloema.

Exceptions in SPE findings:

Nonsecretory Myelomas: *Certain variant of multiple myeloma characterized by an abnormal bone marrow (increased plasma cells) but a normal SPE . They account for account to 1–2% of diagnosed multiple myeloma cases . In such cases, an immunoassay of free light chains (FLC) in serum provides a diagnostic clue toward NSMM which show a significant disproportionate elevation of usually a clone of light chains (kappa or lambda) with an alteration in kappa/lambda ratio (normal Ratio is between 0.60 and 1.65).*

A commonly encountered phenomenon with laboratory testing of FLC includes *"prozone"* effect or *"hook"* effect which occurs due to antigen excess and requires appropriate dilution to obtain reliable results.

Bence-Jones protein estimation in urine is an antique piece of laboratory evidence toward multiple myeloma, which is characterized by detection of light chains in urine. But since the methodology of testing is manual and does not provide standardization, this has been replaced by urine ***Free Light Chain Assay*** in laboratories . Another essential requisite for multiple myeloma work-up includes ***Immunoelectrophoresis.*** The principle employed in immunoelectrophoretic technique involves the use of specific antihuman immunoglobulins (e.g., Anti-IgG, Anti-IgA, AntiKappa, etc.) as a preprocessing step which results in precipitation of immunoglobulins if present and disappearance of the band/peak contributed by that specific immunoglobulin. Hence this technique is also known as ***immunosubtraction.*** This technique aids in typing the specific type of immunoglobulin (including the type of lightchain) contributing to myeloma. This technique is supplemented by quantification of serum immunoglobulins by an immunoassay.

Interpretation hacks :

SPE findings and interpretations:

1. **Acute inflammation early phase ('acute-reaction protein pattern')**

Albumin =–↓, α_1 ↑, α_2 ↑, β =, γ =

2. **Acute inflammation late phase**

Albumin ↓, α_1 ↑, α_2 ↑, β =, γ ↑

3. **Chronic inflammation**

Albumin ↓, α_1 =, α_2 =, β =, γ ↑

4. **Chronic active inflammation**

Albumin ↓, α_1 ↑, α_2 ↑, β =, γ ↑

5. **Malignant tumor**

Albumin ↓, α_1 ↑, α_2 ↑, β =, γ = or ↓ or ↑

6. **Liver failure**

Albumin ↓, α_1 = or ↓, α_2 = or ↓, β =, γ = ↑↑↑

7. **Nephrotic syndrome**

Albumin ↓, α_1 ↓, α_2 ↑↑↑, β ↑, γ ↓

8. **Antibody deficiency**

Albumin =, α_1 =, α_2 =, β =, γ ↓

9. **Pregnancy**

Albumin ↓, α_1 =, α_2 ↑, β ↑, γ =

- ✓ ***In Chronic Liver Disease the γ- fraction is often polyclonal and the normal depression between the β - and γ -band may be missing when liver cirrhosis is present (β – γ bridging).***
- ✓ ***In Hepatocellular Carcinoma when high serum levels of AFP are present, electrophoresis can show a sharp band between the albumin and the α 1 –zone.***
- ✓ ***A marked reduction of the α1- zone is typically due to a deficiency of α 1 –antitrypsin.***
- ✓ ***In inflammatory bowel disease several changes can been noted in electrophoresis, alone or in combination. Impaired absorption from intestinal inflammation may lead to mal assimilation and malnutrition showing a decrease of albumin, and the α 1 - and β-zone on SPE. For the same reasons, iron-deficiency anemia may also be present presenting a localized band in the β-zone. Depending on the activity state of the inflammatory bowel disease, the patterns of inflammation found with electrophoresis can vary from acute inflammatory response in a flare of the disease, to a chronic inflammatory response during stable phases or even a normal pattern when the patient is in remission.***

TABLE 2 : Differential diagnosis of mono- and polyclonal gammopathy

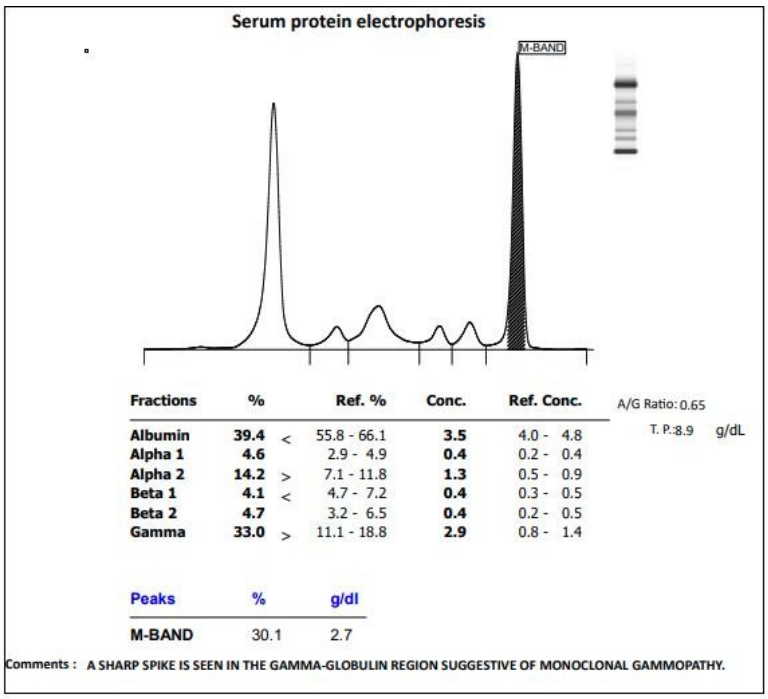
Monoclonal gammopathies
• Multiple myeloma
• Monoclonal gammopathy of undetermined significance
• Smoldering multiple myeloma
• Plasma cell leukemia
• Solitary plasmacytoma
• Waldenström's macroglobulinemia
• Heavy chain disease
• POEMS syndrome
• AL (light chain) amyloidosis
Polyclonal gammopathies
• Infections: viral: hepatitis, HIV, EBV, VZV; bacterial: endocarditis, osteomyelitis, bacteremia, tuberculosis
• Connective tissue diseases: connective tissue disease, temporal arteritis, rheumatoid arthritis, sarcoid
• Liver disease: cirrhosis, ethanol abuse, autoimmune hepatitis, viral-induced hepatitis, PBC, PSC
• Malignancies: solid tumors, ovarian tumors, lung cancer, HCC, renal tumors, gastric tumors
• Hematologic and lymphoproliferative disorders: lymphoma, leukemia, thalassemia, sickle cell anemia
• Other inflammatory conditions: gastrointestinal (CD, UC), pulmonary (bronchiectasis, cystic fibrosis, chronic bronchitis, pneumonitis, endocrine (Graves' disease, Hashimoto thyroiditis)

Limitations:

SPE reports contain Interpretation of the electrophoretic pattern, followed by comments of such an interpretation along with the piece of advice to the clinician if indicated. Each Biochemist / pathologist use his/her own means of interpretation / communication. There is a big lacuna in having a standardized format of reporting SPE for ensuring patient safety and clinician follow-up. There are no international guidelines, however, the Australasian Association of Clinical Biochemists has come out with a standardized format of reporting SPE.

Real life to reel life :

1. A 76-year-old female with a sharp spike is seen in the gammaglobulin region suggestive of monoclonal gammopathy.



Acknowledgement :

The real life cases described above are reported by the doctors of Suraksha Diagnostics Pvt. LTD. Special thanks to my senior colleagues Dr. Neepa Chowdhury, Section Director, Department of Clinical Chemistry, Immunochemistry & Biochemical Genetics. & Dr. Suparba Chakraborty , Consultant Biochemist, Department of Clinical Chemistry, Immuno -chemistry & Biochemical Genetics , Suraksha Diagnostics Pvt. Ltd . for sharing their cases with me for the write up .

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Contributors:
Dr. Anannya Ghosh, Department of Clinical Chemistry, Suraksha Diagnostics Pvt. Ltd., Kolkata
Dr. Neepa Chowdhury, Department of Clinical Chemistry, Suraksha Diagnostics Pvt. Ltd., Kolkata
Dr. Suparba Chakraborti, Department of Clinical Chemistry, Suraksha Diagnostics Pvt. Ltd., Kolkata

Editor & Illustrator:
Dr. Manali Sinha Ray, CNMC, Kolkata
Dr. Abhra Ghosh, DMC&H, Ludhiana