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Comparison of chromatographic method for quantitation of free protein

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ABSTRACT

Chromatography is an important technique for the separation, identification, and purification of components of a mixture for qualitative and quantitative analysis. Protein can be quantitated based on the different properties such as shape and size, total charge and binding capacity. So the different types of the method were present to quantitate the protein. Like Ion Exchange Chromatography (IEC), Size Exclusion Chromatography (SEC), affinity chromatography and reverse phase chromatography. Also in SEC and IEC, we compare the different types of parameters like time, resolution, cost, temperature, detectors and resolution. So comparing these methods we decide that the size exclusion chromatography method was better than the ion exchange chromatography for quantitation of protein molecules.

Keywords— *Size exclusion chromatography, Ion exchange chromatography, Therapeutic protein, Protein aggregates, Biopharmaceuticals*

1. INTRODUCTION

Chromatography is based on the principle where the molecules are separated on the basis of the stationary phase and the mobile phase. The factors are adsorption (liquid-solid), partition (liquid-solid), and affinity or differences of their molecular weights. Because of these difference, the larger molecules come faster through the void volume and smaller molecules come through the total volume.

There are different chromatographic methods was present. Like ion exchange chromatography, affinity chromatography, reverse phase chromatography and size exclusion chromatography. Chromatography is based on the precept wherein molecules in combination carried out onto the floor or into the solid, and fluid desk bound section (strong phase) is separating from every other whilst transferring with the resource of a mobile phase. The factors effective on this separation procedure encompass molecular traits related to adsorption (liquid-solid), partition (liquid-strong), and affinity or variations among their molecular weight. Due to those variations, some components of the aggregate live longer inside the desk-bound segment, and they pass slowly inside the chromatography device, while others pass swiftly into cell phase, and go away the machine quicker.

In chromatography two types of phases are present one was a stationary phase and other was the mobile phase. In stationary phase liquid adsorbed on the surface of solid support and in mobile phase the phase is made up of liquid and gaseous components. Types of chromatography:

(a) Column chromatography
(b) Ion-exchange chromatography
(c) Size-exclusion chromatography
(d) Affinity chromatography
(e) Reverse phase chromatography
(f) Paper chromatography
(g) Thin- layer chromatography
(h) Gas chromatography

For quantitation of protein different chromatographic methods are their like size exclusion, ion exchange chromatography, affinity chromatography and reverse phase chromatography. From these 4 methods, we compare the 2 methods i.e. SEC and IEC.

2. LITERATURE REVIEW

The size-based separation by chromatography was first developed by Synge and Tiselius [1].

Ozlem Coskun in this paper they explained about the different types of chromatographic techniques. In the chromatographic techniques, the separation is carried out on the basis of the charge, molecular weight, partition coefficient and the different phases. So the chromatography was the best methods for the separation of the molecules. And in this paper they explained about all chromatographic methods in detail [2].

Paul Hong described that the SEC method is used for the analysis of therapeutic protein and their aggregates. In that paper, he says that nowadays the use of therapeutic protein was increased. The quantitation of aggregates is of particular concern given a potential effect on the efficacy and immunogenicity. Also, he explained that the SEC for analysis of protein and also instrumentation for these analyses, including

the use of different types of detectors, the effect of mobile phase and column parameters [3].

Gloria Brusotti described that the SEC is a well-established method for the characterization of the molecules and their aggregates. To increase resolution and faster analysis SEC has led the development. Also, he explained that the applications of the SEC [4].
Abraham explained how protein molecule was characterized by the Ion- exchange chromatography [5].

3. METHODS OF CHROMATOGRAPHY

3.1 Size- exclusion chromatography

Size exclusion chromatography is a form of partition chromatography used to separate molecules of different molecular sizes. The basic principle of size exclusion chromatography is relatively simple. Molecules are partitioned between a mobile phase and a porous matrix as phase comprising a porous matrix as a function of their relative sizes. A column constructed of two volumes that is external volume and internal volume. The external volume is usually referred to as the void volume (Vo) while the sum of the external and internal volume is the total volume (Vt). The molecules larger than the pores of the stationary phase matrix will be excluded from the internal volume within the beads. They will, therefore, migrate quite rapidly through the column, emerging at V_o, while molecules smaller than the matrix pores will equilibrate with both external and internal liquid volume, causing them to migrate much more slowly and emerge at a volume (Ve) greater than Vo . Therefore the molecules eluted in order of decreasing molecular size.

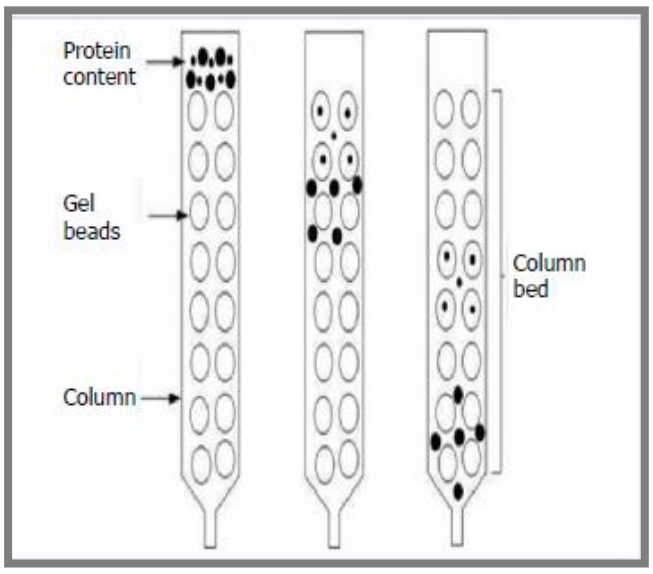

Fig. 1: Gel permeation chromatography

3.2 Ion-exchange chromatography

Ion exchange chromatography may define as a reversible exchange of ions in the solution with ions electrostatically bound to some sort of insoluble matrix or stationary phase.

This technique is useful in the separation of charged compounds like protein and amino acids. Ion exchange chromatography relies on the attraction between the oppositely charged stationary phase, known as ion exchange and analyte.

The ion exchanger consists of an inert support medium coupled covalently to positive or negative functional groups. To these covalently bound functional groups, the oppositely charged ions are bounded, which will be exchanged with charged ions in the sample having charge magnitude more than the ion bonded to the matrix.

Thus if anion exchange chromatography is performed, negatively charged sample components will interact more with the stationary phase and will be exchanged for like charged ions already bounded to the matrix.

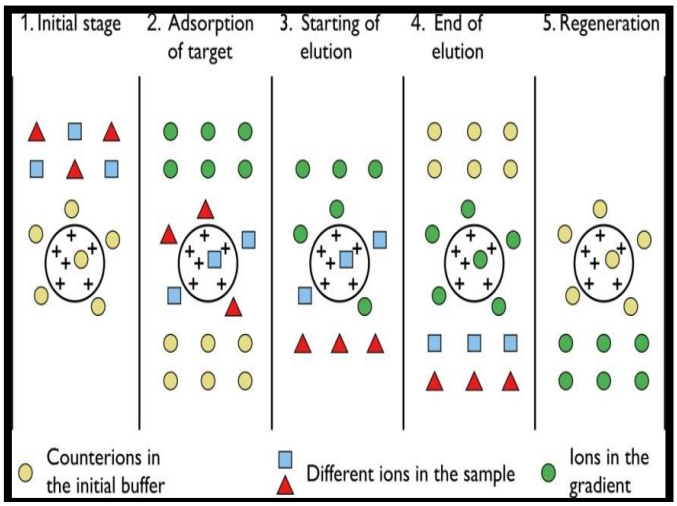

Fig. 2: Ion- exchange chromatography

4. COMPARISION

Table 1: Comparison between SEC and IEC

Parameters	SEC	IEC
Principle	On the basis of size	On the basis of the charge
Time	Less time	Time-consuming
pH	No pH optimization	pH optimization required
Temperature	Less affect the sample	Charges are affected
Resolution	Without parameter optimization, it gives good resolution	Parameters need to optimize
Price	Less price	More price
Detector	UV-VIS,MALS	UV-VIS,Fluroscence, Amperometric
Wavelength	Two-wavelength	Single Wavelength
Validation	Easy to use to validate	Complicated

4.1 Principle

SEC can separate the molecules on the basis of size only, but in IEC the molecules can separate on the basis of the charge,

4.2 Time

The SEC took less time as compare to the IEC because in IEC we need to charge the molecules so it takes a long time.

4.3 pH

In SEC pH optimization was not required, but in IEC pH optimization was required because the particles size greatly influence the resolution.

4.4 Temperature

in SEC the temperature changes are less affect the sample, but in IEC temperature changes were affect the sample because of charges present on the molecules.

4.5 Resolution
In SEC parameters need not optimize, but in IEC parameters need to be optimized.

4.6 Price
SEC takes a less price as compared to the IEC.

4.7 Detectors
In SEC UV-VIS and MALS detectors are used. The MALS detector gives resolution not only the protein molecules but also their aggregates. In IEC UV-VIS, Flurocense and Amperometric detectors are used.

4.8 Wavelength
In SEC we can use two wavelengths at a time, but in IEC we can use only one wavelength at a time.

4.9 Validation
In SEC validation was easy than the IEC, because the broad adoption of this method for these analyses can be attributed to its simplicity, reproducibility, sensitivity and speed. (table 1). So the SEC is the best method for the quantitation of protein than the IEC.

5. ADVANTAGES OF SEC

- The simplicity of the physical separation mechanism
- Easy to validate
- High speed and precision in separation
- Good variety of columns from different manufacturers
- Low levels of aggregation can be measured with a very small amount of material

6. APPLICATIONS

There have been a large number of applications of SEC reported over the last few years. The first application of SEC is monitoring the protein aggregation and quaternary structure in biopharmaceutical industries. SEC considered as the standard method for monitoring protein aggregation. SEC is also used for the development of the purification process for biopharmaceuticals.

The optimization of SEC is ideal due to the short run time. The short run time and quantitative reproducibility make SEC an appropriate method for stability monitoring. These stability protocols provide a good sample throughput. The assurance of the SEC method is providing a valuable part of protein characterization study. SEC also used for polymer synthesis.

7. RESULTS & FUTURE PROSPECTS

SEC is essential chromatography for the determination of the molecular weight of protein and aggregation of the therapeutic proteins. These methods give a higher resolution than the IEC. Also, it takes less time and it requires less price. The validation of the SEC is easy than the IEC.

Sec has been used to guide the manufacturing process and formulation of biotherapeutics. The broad adoption of this method is simplicity, reproducibility, sensitivity and speed.

Sec method development should also include an appropriate evaluation of the chromatographic recovery of both drug product and any aggregates forms present in the samples. Quantitation of protein sample can be estimated by the SEC. It also used for the development and validation of the samples.

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