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Protein (Influenza) identification and quantification by different analytical techniques

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Abstract

Proteins make up more than half the dry weight of the cells. They contribute to the structure of a cell and are obliged for cellular functions analogous as catalysis and molecular recognition. Also, the phenotypes are linked by the proteins not by the genes. The disquisition now a day is going in a direction towards the characterization of the proteome, or the whole set of the proteins set up in the cell, kerchief or organism. To understand how cell works, there is a demand of identification and characterization of the proteins. In short accurate protein quantitation is essential to protein studies in a multitude of disquisition motifs. So that a wide array of different protein assays has been developed to quantify and identify both complex mixtures of proteins as well as a single type of proteins. Total protein quantitation styles include Bradford assay, BCA assay and Absorbance at 280m. Kjeldahl system is used to know the chance of protein present in the sample. There are multitudinous ways for the identification of proteins, mainly there are two ways performed during design work that are SDS - runner and Immunoblotting. Estimation of the total protein attention in bio Medicinals or remedial drugs is truly important. It gives the information about the stability of the drugs. Stability testing is one of the essential ways on the path of drug development. The process determines whether any changes like physical, chemical or microbiological affect the effectiveness and integrity of the final product. Particular fashion is been distributed to different samples depending on the structure of proteins, vacuity of morals and the perceptivity of the fashion. The acceptance criteria for all the mentioned ways were achieved.

Keywords: Protein quantification, proteomics, cellular function, protein assays, stability testing, SDS-PAGE, immunoblotting

Introduction

Proteins are large molecules made of amino acids, which are the basic building blocks of proteins. These amino acids contain carbon, hydrogen, oxygen, and nitrogen. In total, there are 22 standard amino acids that link together to form unbranched polymers. The connections between amino acids are called peptide links, forming polypeptide chains that can have different structures and functions. Proteins have four levels of structural organization: primary, secondary, tertiary, and quaternary, leading to their diverse properties. They are essential in food because they are a key energy source and provide important amino acids that the body cannot produce by itself.

The term "drug" usually involves aspects like specificity, stability, solubility, and safety. There are strict regulatory issues surrounding the development of drugs, particularly vaccines, including checking for purity. The drug manufacturing process includes three key steps: producing experimental batches, submitting an Investigational New Drug notice for clinical testing, and thoroughly assessing the drug's characteristics and testing results before it reaches the market. Quality Control (QC) checks the identity, strength, and purity of drugs to ensure safety and efficacy, as impurities can lead to side effects.

Therapeutic proteins are specially engineered proteins used for medical purposes. Their usage has grown significantly since insulin became the first therapeutic protein in the 1920s. Therapeutic proteins are categorized into three generations, ranging from recombinant versions of natural proteins to gene therapies. They perform various functions such as replacing missing proteins, enhancing biological pathways, and delivering drugs to specific sites within the body.

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Proteomics is the study of proteins, including their identification, expression, and interactions. This field aims to identify changes in protein expression, analyze protein modifications, and understand how protein structure relates to function. Different types of proteomics include expression proteomics, which examines how proteins change under different conditions; structural proteomics, which assesses the three-dimensional shapes of proteins; and functional proteomics, which investigates protein interactions.

Quantifying and characterizing proteins is critical because proteins are involved in nearly all cellular processes. Understanding how proteins work helps in developing safe therapeutic proteins, which can cause serious side effects if not managed properly. Therefore, quantification of protein concentration is essential during drug development. Different techniques can be employed for protein quantitation, and selection depends on the stage of drug development and individual protein properties.

When choosing a protein quantitation technique, one must consider various factors such as the stage of the drug production process, whether total protein content or a specific substance is measured, and the properties of proteins. Common assays for protein quantification include Bradford Assay and Bicinchoninic Acid (BCA) Assay. Techniques for protein identification include SDS-PAGE and immunoblotting.

Successful applications of these methods are evident in areas like oncology, where proteomics aids in understanding cancer and tumor behavior; bio-medical research focusing on infections; agricultural research on plant responses to pests; and food safety by ensuring quality in food products through proteomic analysis.

Protein is a complex substance found in all living organisms, composed of amino acid polymers. In 1902, Emil Fischer and Hofmeister made significant contributions to the understanding of proteins. They are vital for metabolic activities, and research into human genes and proteins aids in identifying potential new drugs for diseases.

The term "proteomics" was introduced by Wilkins in 1995, referring to the large-scale study of all proteins in a cell line, tissue, or organism. Proteomics focuses on protein composition, structures, functions, and interactions, providing a deeper understanding of biological systems than genomics. Proteins usually function by working together, and proteomics aims to identify these interactions. However, studying proteomics is complex because protein expression can vary with time and environmental factors.

Several methods have been developed to quantify proteins, typically categorized into two types based on their chemistry: copper chelation and dye binding. Common techniques for protein quantification include the Bradford assay, BCA assay, measurement of absorbance at 280 nm, and the Kjeldahl method.

The Bradford/Coomassie Brilliant Blue assay, established in 1976, estimates protein concentration by measuring the dye's color change when it binds to protein. This method accurately measures specific amino acids but may not be suitable for all protein concentrations. It has various applications, including measuring protein levels in cerebrospinal fluid and adrenal tissue extracts.

The BCA assay, created in 1985, uses a two-step process where proteins reduce cupric ions to cuprous ions, producing a colored complex measured at 560 nm. This method can quantify proteins even in challenging solutions, such as those

with impurities.

Absorbance at 280 nm is simple and estimates protein concentration based on the UV absorption of aromatic amino acids. It requires a minimal sample volume and is a quick method.

The Kjeldahl method, introduced in 1883, measures nitrogen content to estimate protein. This widely used method in various analyses involves converting nitrogenous compounds into ammonium sulfate and quantifying ammonia.

SDS-PAGE is a technique used in biochemistry to separate proteins by molecular weight. Proteins are denatured and run through polyacrylamide gels in an electric field, allowing for size-based separation. This method is essential for determining protein size and purity.

Immunoblotting, or Western blotting, is a method that uses specific antibodies to detect proteins following SDS-PAGE. This technique allows for qualitative and quantitative analysis of proteins, identifying them with high sensitivity. Choosing the right protein assay depends on compatibility with the sample and the potential interference from other substances. Researchers often use different assays based on their specific needs, considering factors such as speed, volume, and accuracy.

In summary, protein study through proteomics is critical for understanding biological systems and developing new treatments, with various methods available for protein quantification and analysis. Each method has advantages and disadvantages based on sample compatibility and other criteria. Researchers need to select the most suitable method to ensure accurate results.

Materials and Methods

The protein quantification and identification procedures were conducted at the biotechquality control department, r&d ii, cadilapharmaceutical limited, dholka, following the organization's Standard Test Procedure (STP). Various methods were used, including Bradford Assay, BCA Assay, OD at 280nm, Kjeldahl method for quantification, and SDS-PAGE and Western blotting for identification.

For the Bradford Assay, specific reagents were used, including BSA from SIGMA and Bradford's reagent from BIO-RAD. Equipment needed included a multimode plate reader, centrifuge tubes, and micropipettes. To prepare the Bradford reagent, a 1 mg/ml BSA stock solution was made and stored at -20°C. Internal controls and test solutions were mixed properly, and absorbance was measured at 595 nm after incubating the plate. The protein concentration was calculated using a calibration curve plotted in Excel.

The BCA Assay required additional reagents, such as a BSA stock solution from NIST. Similar materials and equipment to the Bradford Assay were used. The protein samples were diluted with DPBS, and a working BCA reagent was prepared. The absorbance was measured at 562 nm after incubating samples at 37°C for 30 minutes. The protein concentration was calculated in the same way as in the Bradford Assay.

For OD at 280 nm, procedures included preparing the UV-Visible Spectrophotometer and using clean cuvettes to measure the absorbance. Protein concentration was determined using the Beer-Lambert law.

The SDS-PAGE method involved various reagents including acrylamide, bromophenol blue, and sodium dodecyl sulfate from SIGMA. Preparation required creating a resolving buffer, stacking buffer, and diluting various components. A

gel casting unit was set up for polymerization, and samples were prepared with either reducing or non-reducing dye. After loading the samples into the gel and running it, a silver staining method was used for visualization. Specific procedures for washing, fixing, and developing the gel were followed to produce clear results.

Immunoblotting involves several key components, including a variety of chemicals, buffers, antibodies, glassware, and equipment. Some of the essential chemicals and their sources are sodium hydrogen phosphate, tris, sodium chloride, Tween 20, skimmed milk, and various others from suppliers like Amersco, Sigma, and Cadila. Important buffers used include transfer buffer, phosphate buffer, phosphate buffered saline, and blocking reagent. Antibodies required in the process include primary and secondary antibodies, specifically rabbit anti-goat IgG conjugates.

The necessary glass and plastic wares include nitrocellulose membranes, Whatman filter paper, cold packs, forceps, and trays. Equipment needed for immunoblotting also includes miniature trans-blotters and -80°C freezers for storing antibodies.

The preparation phase involves several reagents, including making a 10X transfer buffer by dissolving Tris and glycine in water. This buffer can be stored at room temperature. Other buffers, such as 0.5 M phosphate buffer and phosphate buffered saline, are also prepared and can be stored at room temperature. Blocking reagent is made by dissolving skimmed milk in PBST, while primary and secondary antibodies are diluted in skimmed milk before being applied to blots. Additionally, a developing solution is created using DAB and hydrogen peroxide in a phosphate buffer.

During gel preparation, clean casting plates are assembled, checked for leaks, and a 12% resolving gel is prepared by mixing specified amounts of water, acrylamide, Tris, SDS, APS, and TEMED. This gel solution is poured between the plates and allowed to polymerize. A 4% stacking gel is similarly prepared and layered on top, following which the gel is allowed to set with the comb added to form wells.

Samples are prepared by mixing them with a non-reducing dye and heating them. The loading pattern outlines how to place the blank, standards, and samples into the gel wells. Once loaded, the gel is connected to a power supply and run until the dye reaches the gel's end.

For the blot setup, glass plates are removed from the gel, and the gel is soaked in a transfer buffer. A nitrocellulose membrane and filter papers are soaked in the buffer and the membrane is pre-wetted in methanol. The transfer sandwich is assembled carefully to avoid air bubbles, filling the tank with chilled transfer buffer for 90 minutes at a set current.

After transferring proteins to the membrane, a blocking step is conducted using 5% skimmed milk in PBST. The primary antibody is then added and incubated, followed by washing. A secondary antibody is added, followed by additional washes.

To develop the blot, the developing solution is applied and gently stirred. After development, the blot is rinsed, dried, scanned, and stored for result interpretation. Each step, from preparation to development, is crucial for achieving accurate results in Immunoblotting.

Bradford assay Result

Table 1: Loading pattern of Bradford's Assay

	1 (Mg/ml)	2 (Mg/ml)	3 (Mg/ml)	4 (Mg/ml)	5 (Mg/ml)	6 (Mg/ml)	7	8	9	10	11	12
A.	B	B	B	100	100	100						
B.	0.05	0.05	0.05	100	100	100						
C.	0.1	0.1	0.1	100	100	100						
D.	0.2	0.2	0.2									
E.	0.3	0.3	0.3									
F.	0.4	0.4	0.4									
G.	0.5	0.5	0.5									
H.	IC	IC	IC									

10 µl of each Blank, Standards (0.05 - 0.5 mg/ml), Internal Control (IC) and Sample were loaded in triplicates (100 Mg/ml). 200 µl Bradford's reagent was inserted in all the well and kept in Micro plate Reader for 5 minutes at 37°C. The program was set in microplate readersoftware.

The colour development can be seen immediately after the

addition of Bradford's reagent. The colour change from brown to blue with the increasing concentration is being observed and compared with the intensity of colour developed in the sample containing wells.

After adding Bradford's reagent, we can see the colour development as shown in the figure below:

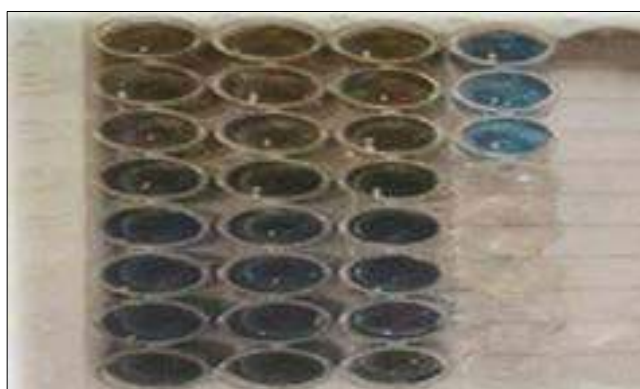


Fig 1: Colour development with the increasing protein concentration in Bradford's Assay

Observe the absorbance of the sample at 595 nm.

Table 2: Obtained Absorbance values of the Blank, Standards, IC and Sample Bradford's Assay

	1	2	3	4	5	6
A.	0.31	0.315	0.3164	0.829	0.8294	0.8365
B.	0.3785	0.38	0.3852			
C.	0.5368	0.5292	0.5004			
D.	0.6632	0.6678	0.6667			
E.	0.7569	0.8066	0.8344			
F.	0.9437	0.9194	0.91			
G.	1.0478	1.0369	1.0302			
H.	0.7745	0.7596	0.7585			

From the results OD of the Blank was subtracted and standard graph of OD of standards was constructed using "MAGELLAN" software. From this concentration can be found for the given sample:

Table 3: Average OD, standard deviation and %CV for different standards in Bradford's Assay

Std Conc. (mg/ml)	Absorbance O.D at 595 nm			Avg. OD	SD	% CV
	Rep 1	Rep 2	Rep 3			
0.5	0.0647	0.0662	0.0714	0.0674	0.0035	5.21
0.10	0.2230	0.2154	0.1866	0.2083	0.0192	9.22
0.20	0.3494	0.3540	0.3529	0.3521	0.0024	0.68
0.30	0.4431	0.4928	0.5206	0.4855	0.0393	8.09
0.40	0.6299	0.6056	0.5962	0.6106	0.0174	2.85
0.50	0.7340	0.7231	0.7164	0.7245	0.0089	1.23

Slope	1.4087
Intercept	0.0442
Regression	0.9857

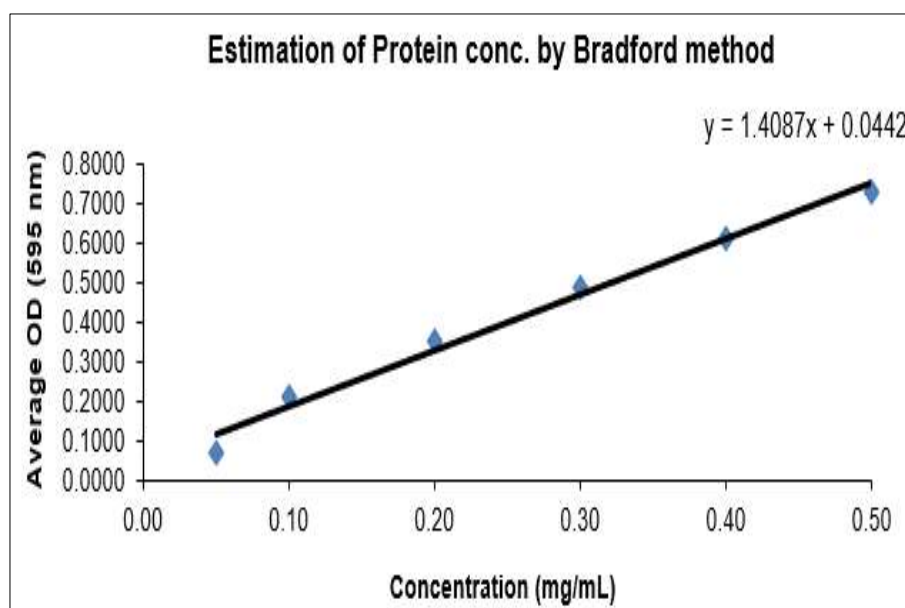


Fig 2: Bradford standard graph - OD v/s Concentration

Table 4: Average OD, standard deviation and %CV for Internal Control in Bradford's Assay

Absorbance O.D at 595 nm			Avg. O.D	SD	%CV	Dilution	Protein(mg/ml)
Rep 1	Rep 2	Rep 3					
0.4607	0.4458	0.4447	0.4504	0.0089	1.98	1	0.288

Table 5: Average OD, standard deviation and %CV for Sample in Bradford's Assay

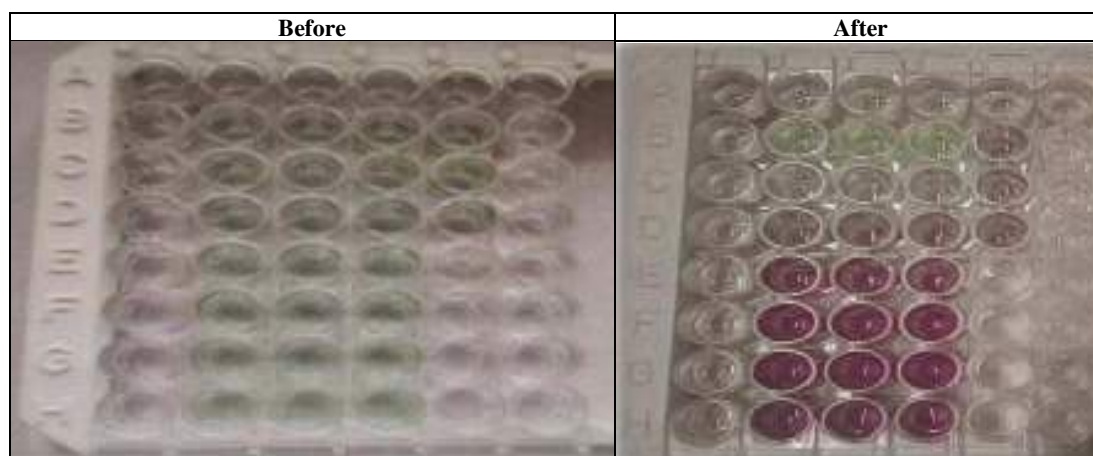
Absorbance O.D at 595 nm			Avg. O.D	SD	%CV	Dilution	Protein (mg/ml)	Mean (mg/ml)
Rep 1	Rep 2	Rep 3						
0.5152	0.5156	0.5227	0.5178	0.0042	0.81	100	33.62	33.62

From the results total protein concentration in sample was 35.39 mg/ml.

BCA assay: Result**Table 6:** Loading pattern of BCA Assay

	1 (Mg/ml)	2 (Mg/ml)	3 (Mg/ml)	4 (Mg/ml)	5 (Mg/ml)	6 (Mg/ml)
A	B	B	B	1	1	1
B	0.1	0.1	0.1	1	1	1
C	0.25	0.25	0.25	1	1	1
D	0.5	0.5	0.5	1	1	1
E	0.75	0.75	0.75	1	1	1
F	1	1	1	1	1	1
G	IC	IC	IC	1	1	1
H	Empty	Empty	Empty			

10 µl of each Blank, Standard, Internal Control and Sample were loaded in triplicates. After that add 200 µl of BCA reagent. Incubated the plate in Microplate Reader at 37°C. The program was set in microplate reader software.

Table 6: Colour development with the increasing protein concentration (Before 30 min. & after 30 min. incubation) in BCA Assay

A purple colour development from green to purple is being seen after 30 minutes incubation. Observe the absorbance of the sample at 562 nm.

Table 7: Obtained Absorbance values of the Blank, Standards, IC and Sample in BCA Assay

	1	2	3	4	5	6
A	0.0923	0.0912	0.0922	0.4586	0.4599	0.4758
B	0.1441	0.1443	0.1488			
C	0.224	0.2292	0.2341			
D	0.3496	0.3607	0.372			
E	0.469	0.4724	0.4984			
F	0.596	0.6086	0.6299			
G	0.3522	0.3592	0.3691			
H	Empty					

Table 8: Average OD, standard deviation and %CV for different standards in Bradford's Assay

Std Conc. (mg/ml)	Absorbance O.D at 562 nm			Avg. OD	SD	%CV
	Rep 1	Rep 2	Rep 3			
0.10	0.0522	0.0524	0.0569	0.0538	0.0027	4.94
0.25	0.1321	0.1373	0.1422	0.1372	0.0051	3.68
0.50	0.2577	0.2688	0.2801	0.2689	0.0112	4.17
0.75	0.3771	0.3805	0.4065	0.3880	0.0161	4.14
1.00	0.5041	0.5167	0.5380	0.5196	0.0171	3.30

Slope	0.5134
Intercept	0.0066
Regression	0.9995

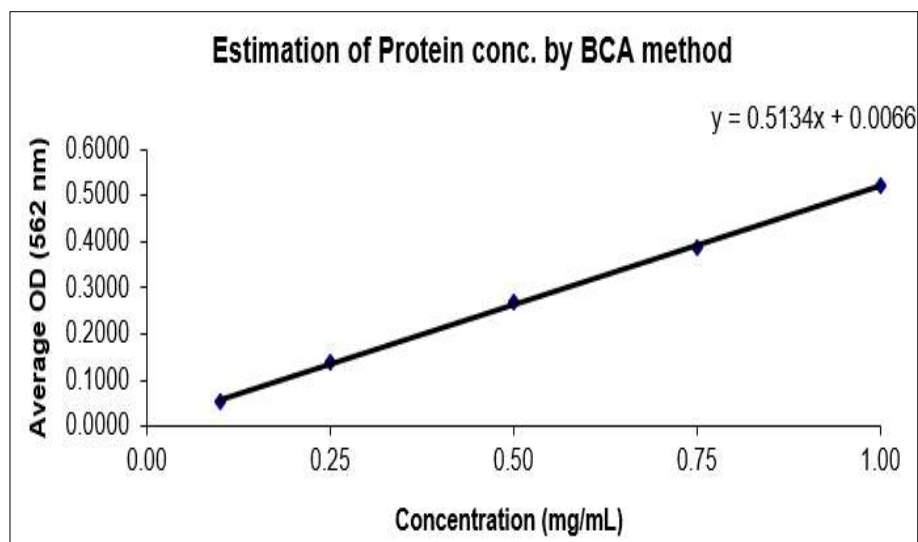


Fig 3: BCA standard graph - OD v/s Concentration

Table 9: Average OD, standard deviation and %CV for Internal Control in BCA Assay

Absorbance O.D at 595 nm			Avg. O.D	SD	%CV	Dilution	Protein(mg/ml)
Rep 1	Rep 2	Rep 3					
0.2603	0.2673	0.2772	0.2683	0.0085	3.1653	1	0.510

Table 10: Average OD, standard deviation and %CV for Sample in BCA Assay

Absorbance O.D at 595 nm			Avg.O.D	SD	%CV	Dilution	Protein (mg/ml)	Mean (mg/ml)
Rep 1	Rep 2	Rep 3						
0.3667	0.3680	0.3839	0.3729	0.0096	2.5685	1	0.714	0.714

From the results total protein concentration in sample was 0.125 mg/ml.

Bradford and BCA assay Comparison

BCA considers the range of protein from 0.1-1.0 mg/ml, Bradford considers the range of protein from 0.05 - 0.5 mg/ml, the absorbance is measured at different wavelength at 562 nm and 595 nm for BCA assay and Bradford assay. BCA is compatible with the detergents, the denaturing agents and the low variability. Bradford is rapid and compatible with the reducing agents.

OD at 280nm

Result: For the absorbance of blank formulation buffer is being used and samples was diluted to 1:5 with the use of formulation buffer itself. All the readings were taken in triplicates and the calculations was done by using mean value and the dilution factor were prepared (1:5).

Table 11: Absorbance of Samples at 280nm

Sr. No.	O.D at 280nm
1	0.2167
2	0.2132
3	0.2153
Mean	0.2150

So, protein content in sample was calculated as given below:

$$\text{Protein content} = \frac{\text{Mean of the OD (0.2150)} \times \text{Dilution factor (i.e. 5)}}{\text{Extinction coefficient (1.53)} \times \text{Pathlength (1 cm)}}$$

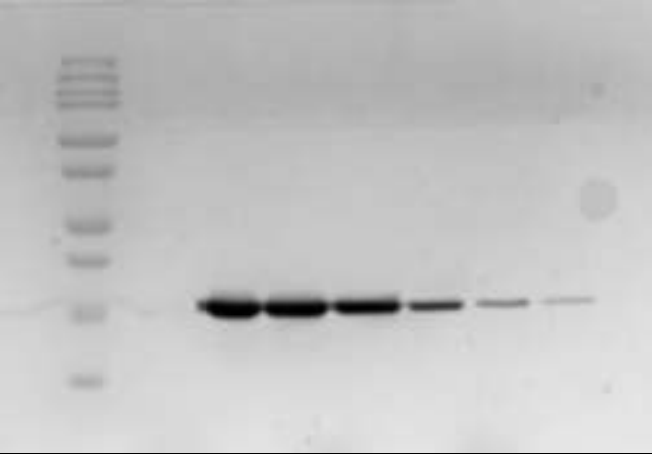
= 0.65 mg/ml So, the sample concentration of protein was 0.65 mg/ml.

SDS-Page

Table 12: Result of Non-Reducing Gel

Sample		Non-Reducing Gel Image	
		1 2 3 4 5 6 7 8 9 10	
Lane 1: Blank	250kd 150kd 100kd 75kd 50kd 37kd 25kd 20kd 15kd 10kd		
Lane 2: Marker			
Lane 3: Blank			
Lane 4: Standard 2µg			
Lane 5: Sample 2µg			
Lane 6: Sample 1µg			
Lane 7: Sample 0.2µg			
Lane 8: Sample 0.04µg			
Lane 9: Sample 0.02µg			
Lane 10: Blank			

Table 13: Result of Reducing Gel

Sample		Reducing Gel Image									
		1 2 3 4 5 6 7 8 9 10									
Lane 1: Blank	250kd 150kd 100kd 75kd 50kd 37kd 25kd 20kd 15kd 10kd										
Lane 2: Marker											
Lane 3: Blank											
Lane 4: Standard 2µg											
Lane 5: Sample 2µg											
Lane 6: Sample 1µg											
Lane 7: Sample 0.2µg											
Lane 8: Sample 0.04µg											
Lane 9: Sample 0.02µg											
Lane 10: Blank											

Western blotting

Table 4.14: Result of Western Blot

Sample		Blot Image			
		1	2	3	4
Lane 1: Molecular weight marker	250kd 150kd 100kd 75kd 50kd 37kd 25kd 20kd 15kd 10kd				
Lane 2: Reference solution					
Lane 3: Sample					
Lane 4: Sample					

Conclusion

From all the experiments and results, the following conclusion can be drawn: All the different samples were checked to find out total protein concentration using different analytical techniques. And the results showed that the final concentration can fulfill the acceptance criteria. Protein quantitation is mainly performed to know the stability of the protein in the drug. The protein quantification was done and pH was measured on the different time intervals to determine whether the product is degrading or not. Various factors like potency, stability and concentration of the protein were done by Bradford assay, BCA assay and Absorbance at 280 nm. Kjeldahl method was efficient to determine the percentage of protein. SDS - PAGE is being carried out exclusively for protein quantification and estimation of protein size and the purity. It is a very efficient technique as it only requires small amount of protein and the migration of the protein can be seen clearly. Western blotting is also a technique that is used for the specific protein. It is done by applying the protein mixture to the gel electrophoresis in a carrier mixture. All the techniques were performed and the results were obtained satisfying the acceptance criteria. This training gave me an exposure to industrial analysis and showcase my theoretical knowledge in the hands-on training.

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