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Method Development and Validation of SDS-PAGE for Quality Control Testing of Pegylated Interferon Alpha-2a

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***Abstract*:** *Hepatitis is one of the most prevailing diseases in Pakistan. Free IFN & Pegylated IFN α-2a is being used to cure chronic hepatitis diseases. A method of impurities determination and molecular weight estimation of PEG-IFN α-2a and Free Interferon α-2a using SDS-PAGE is developed for the purpose of lot release during Quality Control testing. It is the most popular cost-effective method to estimate the molecular weights (MWs) of protein subunits with considerable accuracy.*

In our study method for the identification and estimation of MWs of PEG & Free IFN were validated first before determining MW's and impurities of IFNs from different sources. The R_f values showed consistency in precision. The R_f values were also consistent and comparable in 6 independent samples run by second analyst with CV% less than 0.5%. Beside repeatability and reproducibility, limit of quantification was also determined by two different staining techniques i.e. Coomassie and silver staining. Staining technique using silver ions found more sensitive than Coomassie as it detected up to 0.002µg of protein as compared to 0.05µg detected by Coomassie stain, therefore sliver staining technique is considered more sensitive and preferred technique to estimate impurities. The method has also proved to have resolution power to differentiate between free IFN and PEG-IFN. No affect on method was observed by small variations between gel compositions from 14% to 15%, variation in voltage from 105 to 110V and running buffer pH from 8.6 to 8.8. The analytical method validation showed no interference and thus specific for identification and estimation of molecular weights of free Interferon and Pegylated Interferon.

Thus, the suggested method could be used for routine analysis of free IFN and PEG-IFN for quality control, stability studies and for further studies like western blotting etc.

Keywords: *Free Interferon, Molecular Weight, Pegylated Interferon α-2a, R_f values, SDS-PAGE*

I. Introduction

The determination of absolute, as well as relative purity determination of Pegylated Interferon Alpha-2a (PEG-IFN α-2a) presents substantial challenges of analytical methods, and the results highly depends on method. Therefore for the purpose of lot release an appropriate method should be selected and justified for determination of purity & impurity.

Methods for determination of impurities using SDS-PAGE are available for free Interferon however limited data available for PEG-IFN α-2a. A new proposed method of impurities determination and molecular weight estimation of PEG-α 2a using SDS-PAGE is described here. A number of approaches have been followed to characterize PEG-protein conjugates, among them SDS-PAGE is the most popular method due to its availability, simplicity, reproducibility, comparatively cheaper and ease of use.

SDS-PAGE is widely used to analyze the proteins in complex extracts. The most commonly used methods are derived from the discontinuous SDS-PAGE system first described by Laemmli (1970). The system actually consists of two gels - a resolving gel in which proteins are resolved on the basis of their molecular weights (MWs) and a stacking gel in which proteins are concentrated prior to entering the resolving gel. Differences in the compositions and pH of the stacking gel, separating gel (resolving gel) and electrophoresis buffer produce a system that is capable of finely resolving proteins according to their MWs.

SDS-PAGE is used to identify molecules on the basis of molecular weight and to identify and quantify the impurities of molecular weights. Several blotting techniques are based on SDS-PAGE.

Molecular weight (MW) determination by SDS-PAGE is a dependable method. However an unknown protein's MW should always be obtained by mass spectrometry if a more precise MW estimation is needed. Variations in protein to protein could be minimized by denaturing samples, reducing proteins, normalizing the charge-to-mass ratio and electrophoresing under set conditions. However factors such as protein structure, post translational changes and amino acid composition are variable that are difficult and impossible to minimize and can affect the electrophoretic migration.

The differences can contribute to an error of ±20% when using SDS-PAGE to determine MW of a protein. Despite these limitations SDS-PAGE is still a commonly used method for MW determination of proteins.

SDS-PAGE is a reliable method for determining the molecular weight (MW) of an unknown protein, as the relative front or migration rate of a protein coated with SDS is inversely proportional to the logarithm of its MW. Selecting separation conditions are key to accurate MW determination that produces a linear relationship between log MW and migration within the likely MW range of the unknown protein.

II. Materials & Methods

1.1 Instrument:

SE245 dual gel caster, SE 260 mini-vertical gel electrophoresis unit manufactured by Hoefer Incorporation and EPS 601electrophoresis power supply (6-600V and 1-400mA) manufactured by GE healthcare life sciences was used for electrophoresis.

After application of samples, running buffer was filled to the top and bottom of the reservoir. Any bubbles that were trapped at the bottom of the gel between the glass plates were carefully removed. Electrophoresis was started at constant voltage of 105V except in case of Robustness (Analytical method Validation) where voltage was increased to 110V.

When the dye reached the bottom of the gel or it was disappeared, electrophoresis was stopped. The run was complete when the tracking dye reached the bottom of the gel or it completely disappeared. The running buffer level in the upper buffer chamber was replenished before it falls below the level of the notched plate.

1.2 Reagents:

1.5M Tris-HCl, pH 8.8, 1M Tris-HCl, pH 6.8, Acrylamide/ bisacrylamide (29:1) or 30%, Sodium dodecyl sulfate (SDS)10%, Tetramethylethylenediamine (TEMED), Ammonium persulfate (APS) 10%, Glycine, bromophenol blue, Glycerol, concentrated Hydrochloric acid, β-mercaptoethanol or dithiothreitol (DTT), methanol, ethanol, glacial acetic acid, Coomassie brilliant blue R-250, glutaraldehyde, formalin, silver nitrate, sodium carbonate and sodium thiosulfate were used for resolving gel, stacking gel, running buffer, sample buffer, coomassie satin and silver stain preparation.

Molecular Weight Standard (Markers):

Low molecular weight (LMW-SDS) marker kit, ranges from 14,400 to 97,000Da, manufactured by GE Healthcare Life Sciences was used as standard marker.

1.3 Samples Preparation:

Samples were divided in two groups i.e. set of samples for analytical method validation (AMV) where all samples were reduced before electrophoresis for valid comparison while second set of samples i.e. Free Interferon (Free-IFN) and Pegylated Interferon (PEG-IFN) that were collected from different sources and all samples were tested in both conditions i.e. without reducing and reduced with either mercaptoethanol or dithiothreitol.

1.4 Analytical Method Validation:

Table-1 (Parameters & Acceptance Criteria for AMV)

S.No.	Validation Parameters	Acceptance Criteria
01	**Precision** i Repeatability	%RSD = ≤ 2%
	ii Intermediate precision	%RSD between qualified & second analyst = ≤ 2%
02	**Specificity** i Identification	Results should correspond to standard of specific molecular masses.
	ii Interferences	Placebo and blank should not show any specific band
03	Gradation of intensity	Gradation intensity is seen in electropherogram obtained with reference solution (a) to (e)
04	Limit of quantification	A last dilution of reference solution that gives clear band in electropherogram is the limit of quantification.
05	Resolution	Bands of protein (mixture of samples) with different molecular masses must be resolved and separated out in electropherogram.
06	Robustness	Small but deliberate changes should not affect the comparability criteria of sample with molecular weight standards as mentioned in identification test.

Six samples (precision) of PEG-IFN were prepared by one analyst in a concentration of 0.625mg/mL. 50µL of each sample was applied to prepared gel and run at 105V constant voltage. To measure reproducibility 6

samples (intermediate precision) were prepared by second analyst using same concentration and run in same manner as in case of precision. To observed interference, placebo (excipients used in the product) was applied whether any band appeared. Five samples for gradation of intensity were prepared in concentration 625µg/mL to 1µg/mL, each successive sample was diluted 5 times. Limit of quantification was measured using 6 samples started from 625µg/mL to 0.2µg/mL by diluting samples five-fold each time. One sample intentionally mixed with free Interferon and Pegylated Interferon was also run to measure the resolution power of method used. For robustness deliberate changes applied to observe method sensitivity. These changes include voltage 110V instead of 105V, pH of running buffer 8.8 instead of 8.6 and resolving gel composition 15% instead of 14%.

III. Results & Discussions

SDS-PAGE is a standard method for assessing whether the sample of an isolated protein is identical. SDS-PAGE is also a robust method for the analysis of large supra-molecular complexes. SDS-PAGE denatures and separates individual subunits of these complexes.

In present study method for the identification and estimation of molecular weights (MW) of PEG & Free-IFN were validated first before determining MW's and impurities of Interferons from different sources. The R_f values demonstrated consistency in precision (6 replicates of same sample) **figure-1**. The R_f values were also consistent and comparable in 6 independent samples run by second analyst, **figure-2**. Beside repeatability and intermediate precision or reproducibility, limit of quantification was also determined by two different staining techniques i.e. Coomassie, **figure-3** and silver staining, **figure-4**. Silver staining technique found more sensitive than Coomassie as it detected up to 0.002µg of protein as compared to 0.05µg detected by Coomassie stain, thus sliver staining technique is considered more sensitive and preferred technique to estimate impurities while coomassie staining could be used for estimation of molecular weights. There is clear gradation of intensity observed in both staining techniques i.e. Coomassie staining and Silver staining, **figure-3 & 4**. The method has also proved to have resolution power to differentiate between free interferon and Pegylated interferon, **figure-5**. The method validation showed no interference and thus specific for identification and estimation of molecular weights of free Interferon and Pegylated Interferon, **figure-5**.

Figure-1 (Precision)

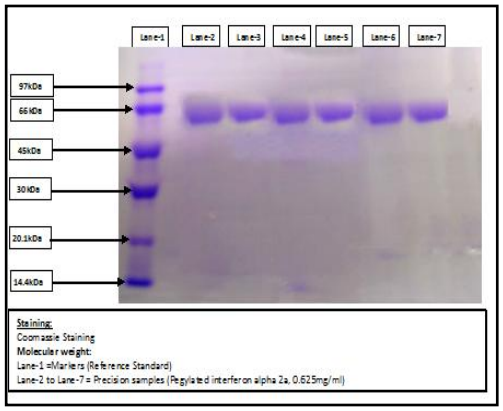
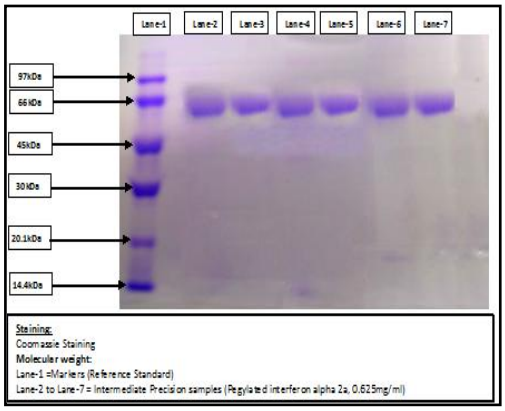


Figure-2 (Intermediate Precision)



Results are repeatable and reproducible as observed in repeatability **figure-1** & intermediate precision **figure-2** performed by second analyst. CV% is less than 0.5% in repeatability while 1.57% between two analysts.

Figure-3 (LOQ using Coomassie Staining)

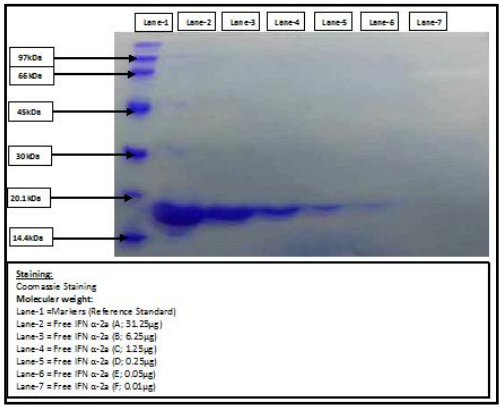
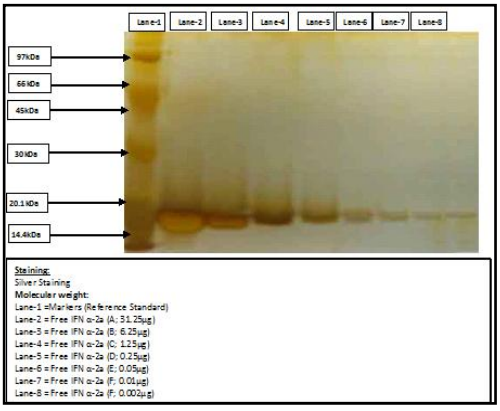


Figure-4 (LOQ using Silver Staining)



Silver staining detected IFN α-2a up to 0.002µg i.e. 25% more sensitive than coomassie staining as shown from lane-8, as very clear band is observed. Thus this technique is suitable for impurities estimation up to 0.002µg.

Figure-5 (Resolution, Specificity and Interference)

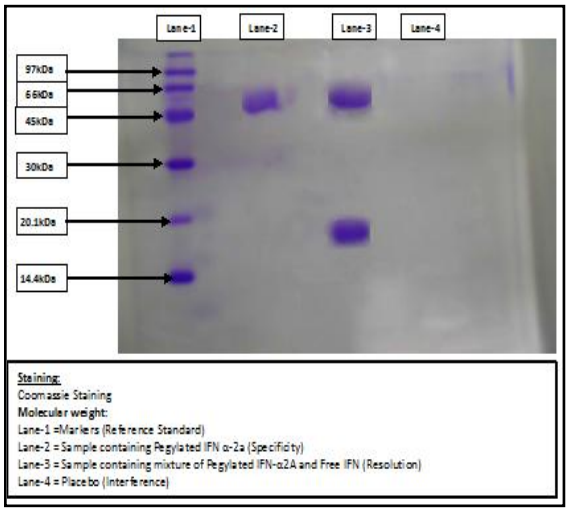
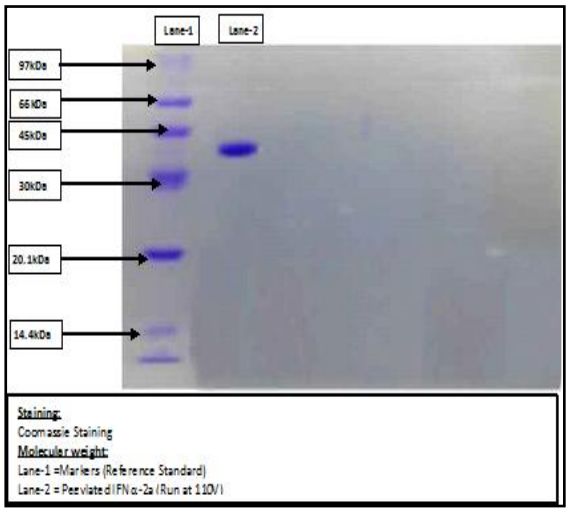


Figure-6 (Robustness using 110V)



From lane-3, **figure-5** it is obvious that the technique could resolve simple mixture easily. Two different IFN moieties i.e. Pegylated IFN (40kDa) and Free IFN (20kDa) separated apart.

The method is not affected by small variations between gel compositions from 14% to 15%, variation in voltage from 105 to 110V and running buffer pH from 8.6 to 8.8, thus analytical method for estimation of molecular weight and impurities is also robust, **figure-6, 7 & 8**.

Figure-7 (Robustness using 15% Gel)

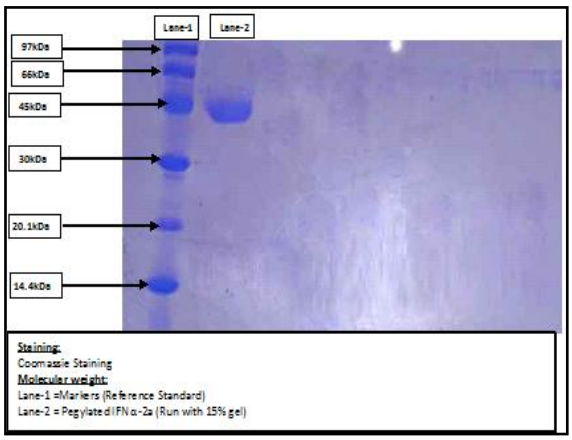
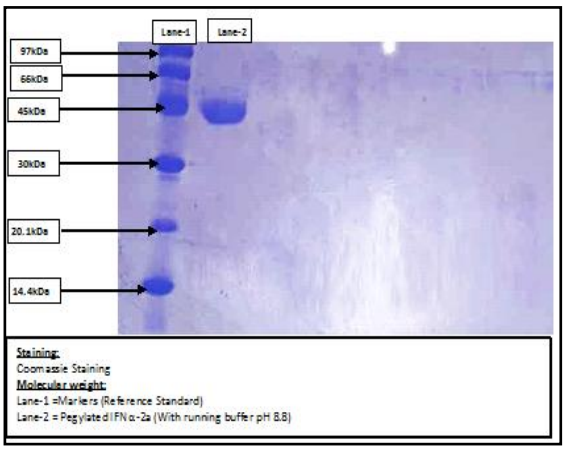


Figure-8 (Robustness using Running Buffer pH 8.8)



Results of all samples collected from different sources showed band correspond to that of reference standard weight. All samples were prepared in 2X diluted sample buffer and were applied in two conditions i.e. directly after preparation and after reducing with dithiothreitol or β-mercaptoethanol. Samples were applied in concentration of 15.625µg in gel. No any additional impurity observed in any of the samples of free Interferon and Pegylated Interferon. Thus after analytical method validation samples from different sources were also analyzed and found compatible with the method used.

IV. Conclusion

Molecular weight (MW) determination by SDS-PAGE is a dependable method. However an unknown protein's MW should always be obtained by mass spectrometry if a more precise MW determination is needed. Protein to protein variation can be minimized by denaturing samples, reducing proteins, normalizing the charge-to-mass ratio and electrophoresing under set conditions. However factors such as protein structure, post translational changes and amino acid composition are variable that are difficult and impossible to minimize and can affect the electrophoretic migration.

The differences can contribute to an error of ±20% when using SDS-PAGE to determine MW of a protein. Despite these limitations SDS-PAGE is still a commonly used method for MW determination of proteins.

The present work refers to the fact that the accurate, precise, and robust SDS-PAGE method was developed and validated for estimation of molecular weights of free and Pegylated Interferon in pharmaceutical bulk intermediate and finished dosage form. The analytical method was validated and found to be simple, accurate, and precise. The recovery percentages showed that the method is free from interference of the excipients used in the formulation. Further method can estimate molecular weights in the ranges from 14 to 97kDa and in the concentrations from 31µg to 0.002µg/50µl. Therefore, the proposed method can be used for routine analysis of free and Pegylated Interferon for quality control testing, stability studies and for further studies like western blotting etc. The method is not affected by small variations between gel compositions from 14% to 15%, variation in voltage from 105 to 110V and running buffer pH from 8.6 to 8.8.

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