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Evaluation of SDS-PAGE for Quality Control Testing of Human Albumin Preparations – A Comparative Study between SDS-PAGE and Horizontal Zone Electrophoresis

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Abstract:

Human Albumin is known as a therapeutic agent since decades and is prescribed for treating patients of various conditions like trauma, haemorrhage, acute liver failure or hypoalbuminemia, shocks, injuries, etc.As albumin is used in critical care therapy, the quality of the product being infused should be considered. Purity of the final product is the most critical factor in pharmaceutical industry and also for patient safety. A method of impurities determination in Human Albumin samples using SDS-PAGE is evaluated for the purpose of batch release or Quality Control evaluation.The results of our study shown that SDS-PAGE method using calibrated densitometer for analysis can be an alternate for the existing Horizontal Zone Electrophoresis (HZE). The molecular weight marker Protein Standards in the range of 10 – 250 KDwere run along with Human Albumin for Electrophoresis BRP 3 (Code: H0900000, EDQM, France) International reference standard (IRS) and Human Albumin samples. SDS-PAGE is already being used as an effective tool for identification purpose of various Biologicals; such as various recombinant products like Human Coagulation Factor VIII (rDNA), PEG & Free IFN, etc. Moreover, the detection limit of amido black stain used in Horizontal Zone Electrophoresis is >100ng and Coomassie Blue in SDS-PAGE can detect even 100ng protein band. The detection sensitivity of Coomassie Blueis higher than that of Amido Black. The results for 25 human albumin samples on statistical analysis using GraphPad Prism software shown non-significant for determination of both principal and other bands with t value 0.98 and 0.53 respectively. Repeatability and reproducibility is also determined with %CV of 0.62 along with 98.64 %Accuracyfor IRS. The method has also proved to have resolution power to differentiate between Human Albumin and Immunoglobulin (Fig.9). In addition to its advantages like sensitive, robust, easily availablereagents,time saving,easy to perform and dependable, SDS-PAGE is found replaceable to existing Protein Composition by Horizontal Zone electrophoresis for quality control evaluation of human albumin samples. Hence, SDS-PAGE with calibrated densitometry method can be added as an alternate to existing Horizontal Zone electrophoresis in Pharmacopoeia for determination of protein composition in human albumin preparations prescribed for various therapeutic treatments.

Keywords*: Human Albumin, Quality Control, Purity, SDS-PAGE, Protein Composition, Horizontal Zone Electrophoresis*

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I. Introduction

Human Albumin is known as a therapeutic agent since decades and is prescribed for treating patients of various conditions liketrauma, haemorrhage, acute liver failure or hypoalbuminemia, shocks, injuries, etc.[1, 2]. As albumin is used in critical care therapy, the quality of the product being infused should be considered. Purity of the final product is the most critical factor in pharmaceutical industry and also for patient safety. Cold Ethanol Fractionation is the traditional method for production of Human Albumin from human plasma[3, 4]. From then, many manufacturers have added additional purification steps to make the final product safe for human use[5-7].

Quality control testing of finished product is mandatory for manufacturers before submission for regulatory approval. These regulatory testing laboratories conduct extensive quality control testing on each and every batch submitted to them as per the relevant pharmacopoeiamonograph to ensure product's quality.

Determination of purity and/or impurity is one of the critical test parameters for quality control testing of human albumin. One of the tests for this purpose is determination of protein composition by Horizontal Zone

Electrophoresis (HZE) using barbital buffer and cellulose acetate membrane strips as recommended in Pharmacopoeia Monograph for Human Albumin (IP/EP/BP)[8-10]. SDS-PAGE is a commonly used technique for analysing purity of proteins, especially in the field of quality control of Biopharmaceutical proteins[11],but its use as an alternate to HZEfor determining the purity in quality control testing of finished product of human albumin is not evaluated and not mentioned in any pharmacopoeia. Limited data available for use of SDS-PAGE for this purpose in research and development phase of various Biologicals but no data available for its use in quality control testing of finished product[12]. The suitability of SDS-PAGE as an alternate to test for protein composition by HZE for quality control evaluation of human albumin is described here. Quantitation of Principal and other bands obtained using densitometer and related software done during the study to express the results in percentage. Thus, this technique also helps to quantify the principal band to assess the purity in addition to its identification. It is well known that SDS-PAGE is the most popular and widely used method to identify molecule on the basis of molecular weight and quantify the impurities due to its simplicity, reproducibility, comparatively cheaper, dependable and ease of use[13, 14]. The most commonly used methods are derived from the discontinuous SDS-PAGE system first described by Laemmli (1970)[15]. The system consists of two gels – a resolving gel in which proteins are resolved on the basis of their molecular weights and a stacking gel in which proteins are concentrated before entering into the resolving gel.

Protein composition by horizontal zone electrophoresis (HZE) technique separates the biochemical molecules in a liquid form. Joachim Kohn (German chemist) in 1957developed cellulose acetate electrophoresis which involves the separation of molecules on membrane or strips of cellulose acetate[16]. Cellulose acetate electrophoresis allows separation of test samples on the basis of molecular sieving effect; here samples move under electric current and separates according to their charge rather than their molecular size. On completion, different components of the sample separate into various bands or zones. Each band represents constituents in the samples that possess similar or identical characteristics[17]. Cellulose acetate possesses larger pores than other popular electrophoretic matrices such as agarose and polyacrylamid[18,19]. The *sieving* influence that cellulose acetate imposes onto the separating components, therefore, is very little or nil. The mobility of different components in the sample is largely based on the overall charge than the size of the component in question in cellulose acetate electrophoresis [16,20,21]. For this reason, the ability to distinguish different components that are very similar in size, or the *resolution* of a cellulose acetate electrophoresis, is rather limited, when compared to gel electrophoresis. Barbital Buffer which is generally used as a running buffer in horizontal zone electrophoresis is a scheduled IV controlled drug. Hence, availability of these chemicals is very stringent for purchasing.

Results of both the HZE and SDS-PAGE are similar as depicted by the present study. From the present study data it can be seen that the results of SDS-PAGE – [Method 'A'] are as good as that of Horizontal Zone Electrophoresis - [Method 'B']. Hence, the same can be used as a promising alternate method for determining the impurities/ purity/ protein composition in Quality Control testing of Human Albumin samples.

II. Materials

A. Material For SDS-PAGE (METHOD 'A'):

Reagents: Acrylamide/bis-acrylamide solution (30%). Resolving (separating) gel buffer (1.5 M Tris-HCl, pH 8.8), Stacking gel buffer (0.5 M Tris-HCl, pH 6.8), Tris Glycine SDS running buffer, Ammonium per sulphate (10%), Sodium Dodecyl Sulphate (10%), TEMED (Tetra Methylene Diamine), 0.9% Normal Saline, Comassie Brilliant Blue R-250 are used in the test.

Molecular weight markers: Precision Plus Protein Standards All Blue Prestained Protein Standards in the range 10 – 250 KD (Bio-Rad) was used as molecular weight markers.

Reference material: Human Albumin for Electrophoresis BRP 3 (Code: H0900000, EDQM, France) was used along with samples to verify the test validity. Human Normal Immunoglobulin for Electrophoresis BRP 3 (Code: H1000000, EDQM, France) was used along with samples to verify the resolution.

Equipment: Mini-PROTEAN Tetra Cell Vertical Electrophoresis, Mini-PROTEAN Spacer Plates with 1.5 mm Integrated Spacers, Mini-PROTEAN Combs and PowerPac Basic Power Supply of Bio-Rad were used for electrophoresis. GL583 MODEL Gel Dryer Apparatus (Bio-Rad); GS- 800 Calibrated Densitometer with Image-lab software(Bio-Rad).

PROCEDURE:10% resolving gel and 5% stacking gel are prepared. Twenty five various preparations (5%, 20% & 25%) of Human Albumin samples from different manufacturers were used in the study. Human AlbuminSamples and reference material are diluted to 0.025% protein concentration (0.25 mg/ml) in 0.9% saline. All samples are reduced with β-Mercaptoethanol.Run started at constant current of 15Watts and stopped the run when dye reached the bottom of resolving gel (approx..40- 45 mins). Comassie Brilliant Blue R-250 (Bio-Rad) was used for staining.The Gel was dried using Gel Dryer Apparatus (Bio-Rad) and analysed using Calibrated Densitometer with Image-lab software(Bio-Rad).Working Mechanismof gel analysis using Densitometer and Imagelab software isdescribed in Fig.1.

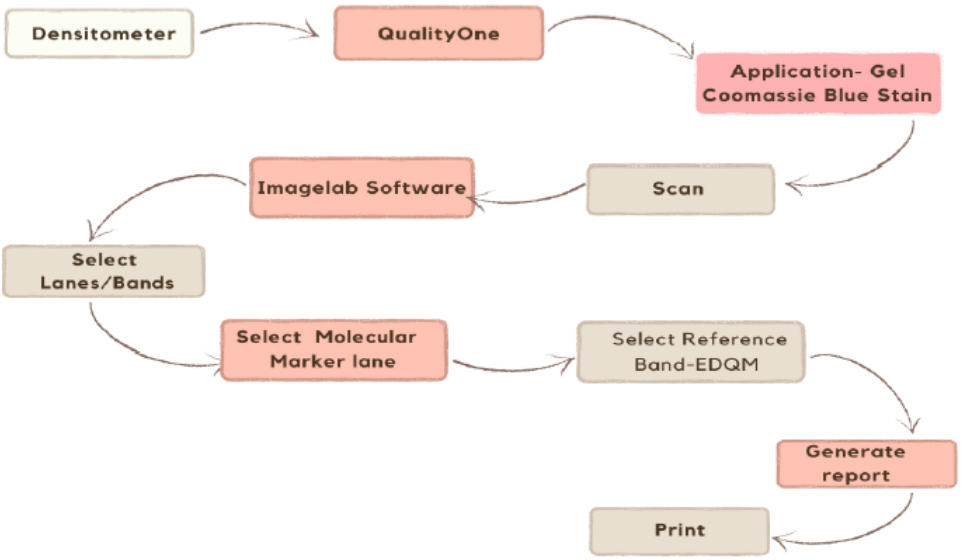


Fig.1- Analysis of gel using Densitometer and Imagelab software

B. MATERIALS FOR HORIZONTAL ZONE - ELECTROPHORESIS (METHOD 'B'):

Protein Composition by Horizontal zone electrophoresis (as prescribed in IP Monograph for Human Albumin) is done for all the Human Albumin samples using cellulose acetate strips and barbital buffer.

Reagents: Barbital Buffer; (Sigma), CellasGel cellulose acetate membrane strips (2.5 x 14cm); (Cleaver Scientific Ltd., UK), Amido Black Stain and normal saline (0.9% NaCl) were used.

Reference material: Human Albumin for Electrophoresis BRP (Code: H0900000, EDQM, France) was used along with samples to verify the test validity.

Equipment: Cellas Electrophoresis system with casting bridge, sample applicator (Cleaver Scientific Ltd., UK), along with power pack and Scanner (EPSON PERFECTION V500 PHOTO) were used for performing Protein composition by horizontal zone electrophoresis.

PROCEDURE: Reference Material,Human Albumin for Electrophoresis BRP (Code: H0900000, EDQM) was used as reference standard in every run along with samples. 2% protein concentration of sample and Reference material were prepared in 0.9% Normal Saline. The electrophoresis run was started at a constant voltage of 175V/ 50W power and run the samples for 45 minutes so that the sample applied on the strip migrates at least 30 mm. Then the strips were gently removed from the bridge and stained with amido black staining solution for 2-3 minutes, followed by destaining. After destaining the strips were scanned using a scanner attached to PC and the resultant bands were analysed using Turbo Scan software to find the percentage of Principal and other Bands obtained in the sample. Results were calculated by taking the mean of three readings of each strip.The same twenty five preparations (5%, 20% & 25%) of Human Albumin samples from different manufacturers along with reference preparation from EDQM, France were used in the study. Working mechanism of Turboscan software is described in fig.2

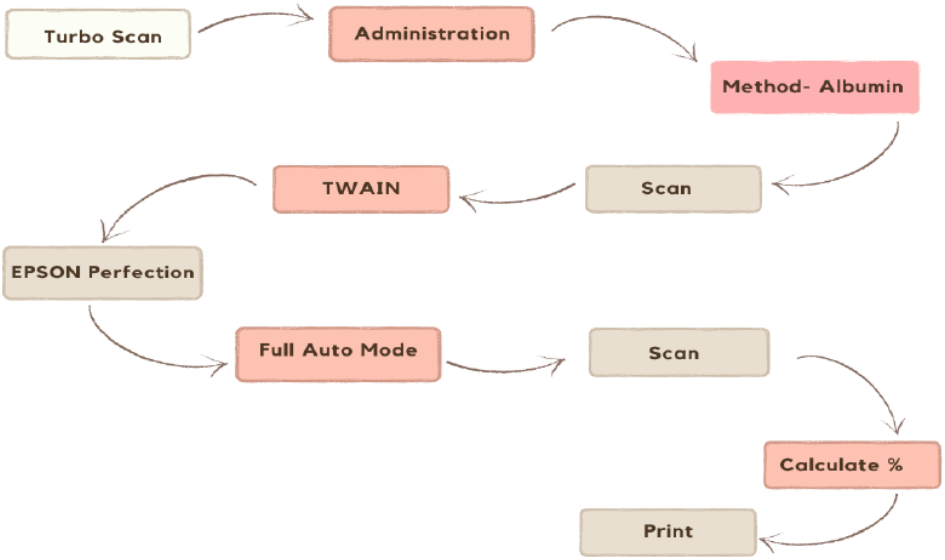


Fig. 2 - Analysis of Cellulose acetate strips using Turboscan software.

III. Results:

25 different samples of Human Albumin (5%, 20% & 25%) were analysed by SDS-PAGE and Densitometer using Imagelab software on different days. International reference standard recommended for Protein Composition by EDQM, France (Human Albumin Reference Standard EP BRP; Batch-3; Code-H0900000;Acceptance Criteria-93.8%-98.3%) was also run in each test along with samples to verify the test validity.ImagesofHZE and SDS-PAGE for one performance are shown in Fig.3 and Fig.4 for illustrative purpose. Results for reference standard are obtained within range (93.8%-98.3%) in every test (Fig.5). All 25 samples are analysed three times and the results obtained w.r.t. Principal and other bands in comparison to that of HZE are summarized in Table 1 and Table2.The molecular weight of Human Albumin in all 25 samples and Reference standard was also observed in the range of 64.6 -69.6kDa as summarized in Fig.6.

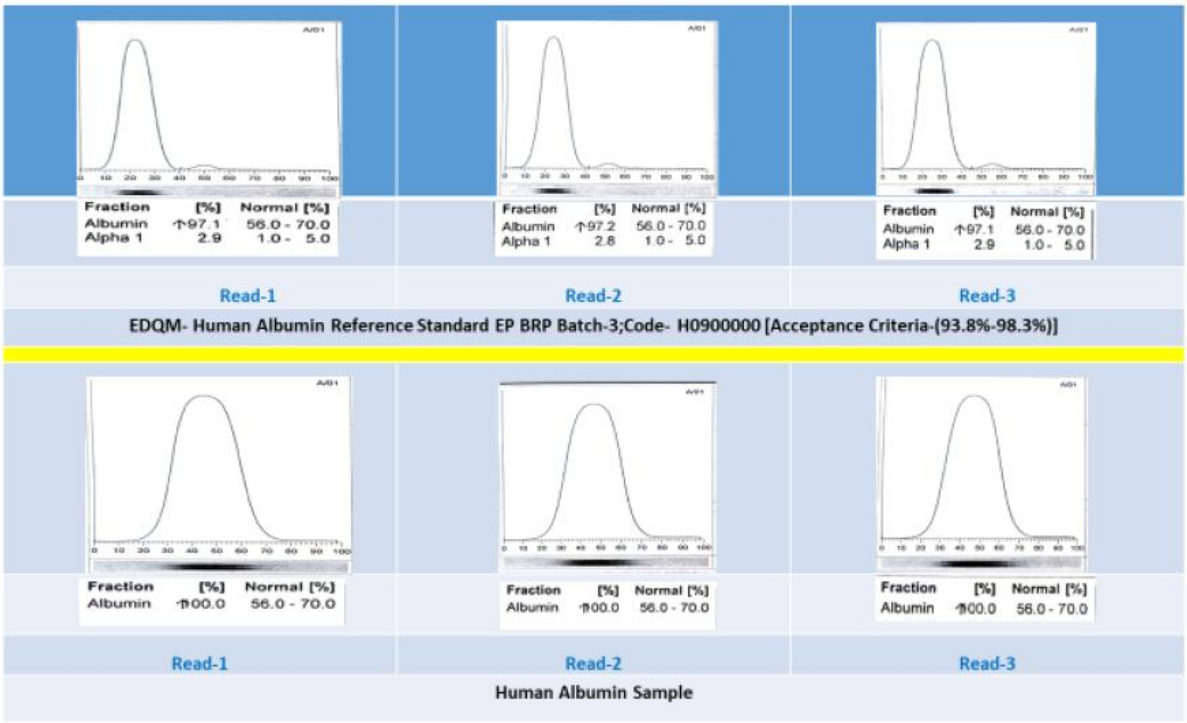


Fig.3: Illustration of - Cellulose acetate electrophoresis (HZE) Protein Composition in Human Albumin Samples (Image of chromatogram obtained by TurboScan Software - one performance is given here for illustrative purpose)

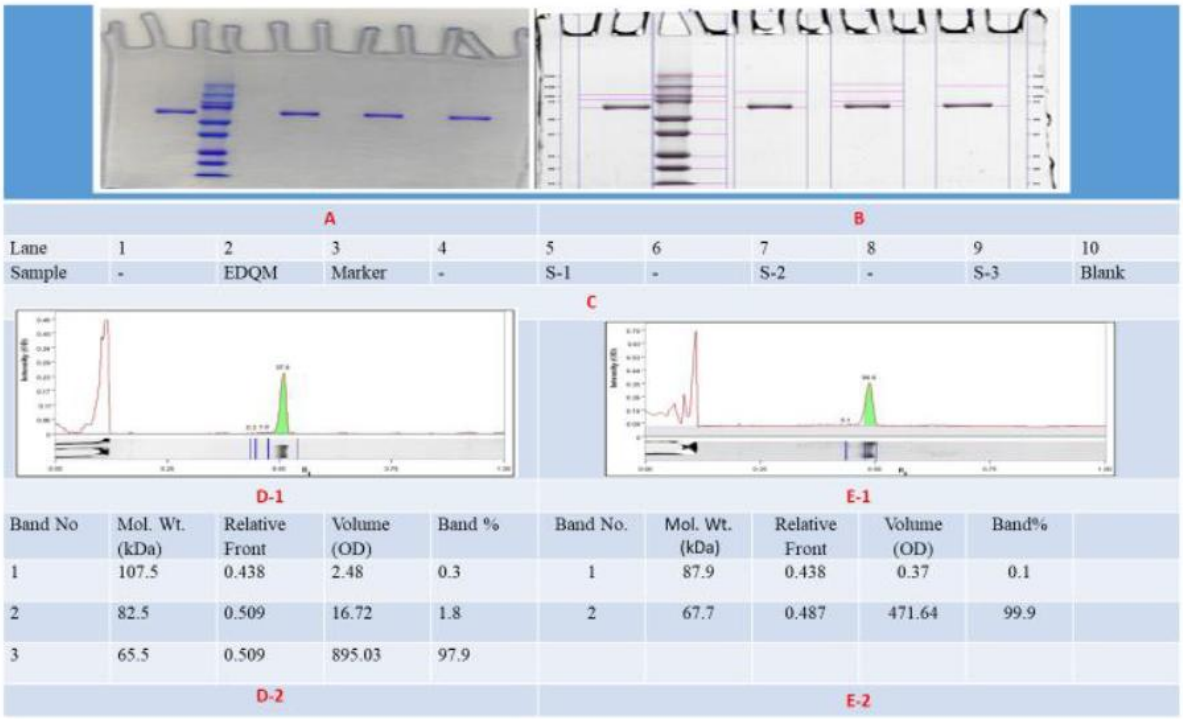


Fig.4: Illustration of Gel (SDS-PAGE) analysis (Image of only one performance is given here for illustrative purpose)A: SDS-PAGE GEL; B: Scanned image of Gel; C: Gel Map.D-1 & D-2: Analysis of Sample (EDQM RS) in lane 2; E-1 & E-2: Analysis of Sample (Human Albumin) in lane 5.

Table 1:Percentage of Principal band: Comparison between Protein Composition by HZE and SDS-PAGE of Human Albumin

S.No.	Sample No.	Principal Band							
		% obtained by HZE				% obtained by SDS-PAGE			
		1	2	3	**Mean**	1	2	3	**Mean**
1	Sample -1	97.5	97.6	97.6	**97.6**	97.5	97.6	97.5	**97.5**
2	Sample -2	96	96.2	96.7	**96.3**	96.3	96.4	96.4	**96.4**
3	Sample -3	98.2	98.2	98.2	**98.2**	98.3	98.2	98.3	**98.3**
4	Sample -4	99.8	99.8	99.7	**99.8**	99.8	99.8	99.7	**99.8**
5	Sample -5	96.2	96.2	96	**96.1**	96	96.1	96.3	**96.1**
6	Sample -6	97.3	97	97.3	**97.2**	97.4	97.4	96.6	**97.1**
7	Sample -7	100	100	100	**100.0**	99.8	99.7	99.7	**99.7**
8	Sample -8	96.5	96.9	97	**96.8**	97	96.8	96.9	**96.9**
9	Sample -9	98.5	98.9	98.5	**98.6**	98.8	98.4	98.7	**98.6**
10	Sample -10	100	99.7	99.9	**99.9**	99.9	99.7	99.9	**99.8**
11	Sample -11	97.1	96.8	96.8	**96.9**	96.7	96.9	96.9	**96.8**
12	Sample -12	97.6	97.3	97.5	**97.5**	97.7	97.7	97.8	**97.7**
13	Sample -13	96.9	97.1	97.2	**97.1**	97.2	97.1	97.3	**97.2**
14	Sample -14	99.5	99.5	99.5	**99.5**	99.7	99.6	99.7	**99.5**
15	Sample -15	99.6	99.7	99.6	**99.6**	99.5	99.5	99.7	**99.6**
16	Sample -16	99.9	99.6	99.7	**99.7**	99.8	99.7	99.7	**99.7**
17	Sample -17	96.6	96.9	96.6	**96.7**	96.8	96.2	96.7	**96.6**

18	Sample -18	99.9	100	100	**100.0**	99.7	99.9	99.8	**99.8**
19	Sample -19	97.9	98.1	97.9	**98.0**	97.9	99.5	98	**98.5**
20	Sample -20	100	100	100	**100.0**	99.3	99.5	99.3	**99.4**
21	Sample -21	100	100	100	**100.0**	99.5	99.7	99.7	**99.6**
22	Sample -22	100	100	100	**100.0**	99.9	99.9	99.9	**99.9**
23	Sample -23	100	100	100	**100.0**	99.8	99.5	99.6	**99.6**
24	Sample -24	99	99.2	99.5	**99.2**	99.4	99.4	99.2	**99.3**
25	Sample -25	97.1	97.2	97.3	**97.2**	97.4	97.3	97.4	**97.4**

Table 2: Percentage of other bands:Comparison between Protein Composition by HZE and SDS-PAGE of Human Albumin

S.No.	Sample No.	**Other bands**							
		% obtained by HZE				**% obtained by SDS-PAGE**			
		1	2	3	**Mean**	1	2	3	**Mean**
1	Sample -1	2.5	2.4	2.4	**2.4**	2.5	2.4	2.5	**2.5**
2	Sample -2	4	3.8	3.3	**3.7**	3.7	3.6	3.6	**3.6**
3	Sample -3	1.8	1.8	1.8	**1.8**	1.7	1.8	1.7	**1.7**
4	Sample -4	0.2	0.2	0.3	**0.2**	0.2	0.2	0.3	**0.2**
5	Sample -5	3.8	3.8	4	**3.9**	4	3.9	3.7	**3.9**
6	Sample -6	2.7	3	2.7	**2.8**	2.6	2.6	3.4	**2.9**
7	Sample -7	0	0	0	**0**	0.2	0.3	0.3	**0.3**
8	Sample -8	3.5	3.1	3	**3.2**	3	3.2	3.1	**3.1**
9	Sample -9	1.5	1.1	1.5	**1.4**	1.2	1.6	1.3	**1.4**
10	Sample -10	0	0.3	0.1	**0.1**	0.1	0.3	0.1	**0.2**
11	Sample -11	2.9	3.2	3.2	**3.1**	3.3	3.1	3.1	**3.2**
12	Sample -12	2.4	2.7	2.5	**2.5**	2.3	2.3	2.2	**2.3**
13	Sample -13	3.1	2.9	2.8	**2.9**	2.8	2.9	2.7	**2.8**
14	Sample -14	0.5	0.5	0.6	**0.5**	0.3	0.4	0.3	**0.3**
15	Sample -15	0.4	0.3	0.4	**0.4**	0.5	0.5	0.3	**0.4**
16	Sample -16	0.1	0	0	**0.03**	0.2	0.3	0.3	**0.3**
17	Sample -17	3.4	3.1	3.4	**3.3**	3.2	3.8	3.3	**3.4**
18	Sample -18	0.1	0.4	0.3	**0.3**	0.3	0.1	0.2	**0.2**
19	Sample -19	2.1	1.9	2.1	**2.0**	2.1	0.5	2.0	**1.5**
20	Sample -20	0	0	0	0	0.7	0.5	0.7	**0.6**
21	Sample -21	0	0	0	0	0.5	0.3	0.3	**0.4**
22	Sample -22	0	0	0	0	0.1	0.1	0.1	**0.1**
23	Sample -23	0	0	0	0	0.2	0.5	0.4	**0.4**
24	Sample -24	1	0.8	0.5	**0.8**	0.6	0.6	0.8	**0.7**
25	Sample -25	2.9	2.8	2.7	**2.8**	2.6	2.7	2.6	**2.6**

The mean and SD for Principal band of reference material used (EDQM, France) by SDS-PAGE are 97.356 & 0.612 respectively. The same by HZE are 97.016 and 0.727 respectively. Percentage of Principal band observed by both methods is represented graphically in Fig.5. The two-tailed P value for other bands

equals to 0.1095, this difference is also considered to be not statistically significant. t= 1.6623 at 95% confidence interval and the standard error of difference obtained is 0.205. Results observed for Molecular weight of all 25 samples along with EDQM reference preparation are also statistically analysed similarly. The two-tailed P value in this case equalsto 0.0728 which is also not quite significant statistically. Same is shown graphically in Fig.6. The mean of samples minus EDQM is 0.604 and t=1.8764 at 95% confidence interval.

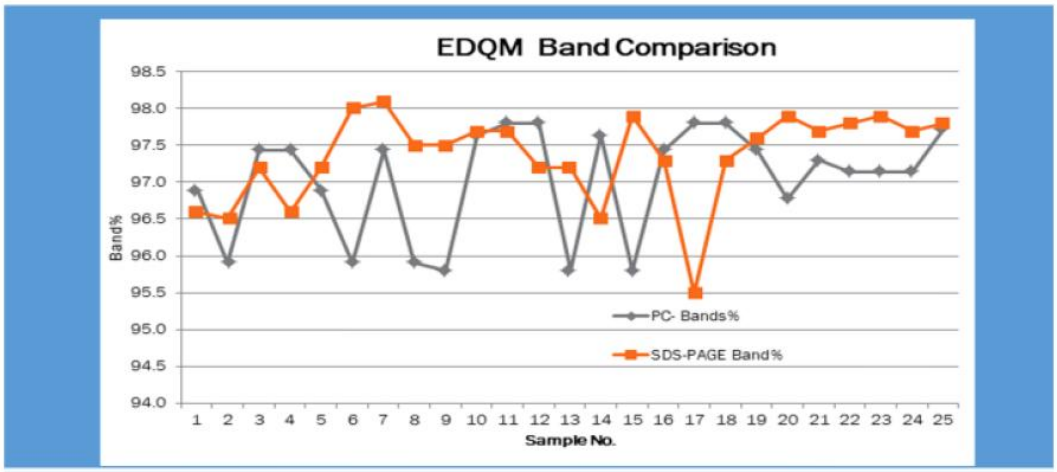


Fig.5: Principal Band values observed for EDQM- Reference standard by SDS-PAGE & Zone Electrophoresis

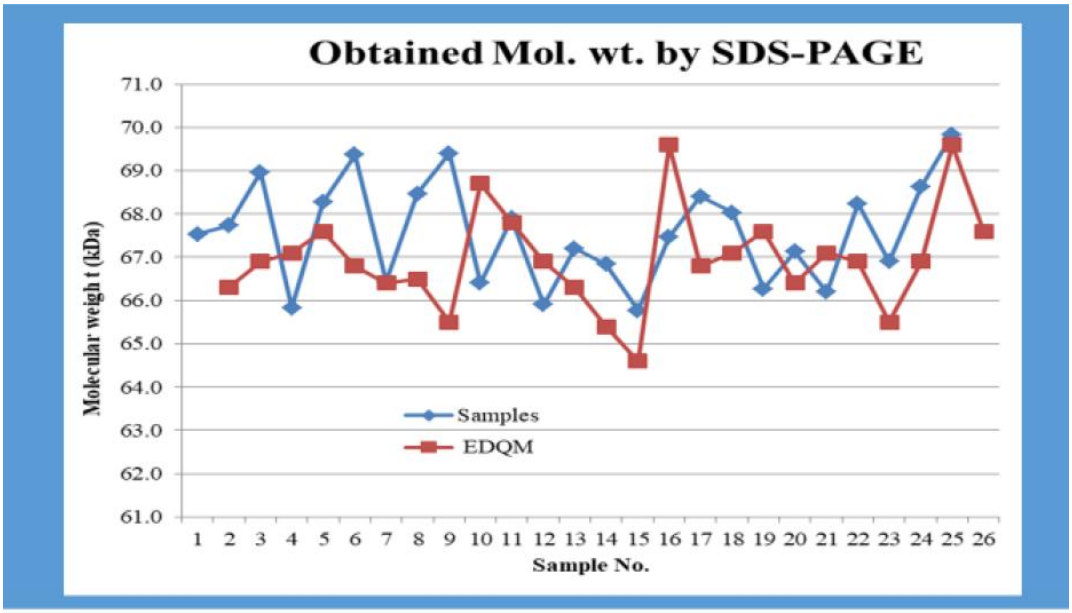


Fig.6: Molecular weight of samples & EDQM observed by SDS-PAGE

The results were analysed statistically using GraphPad Prism Software. Percentage of principal band observed by both methods for 25 samples is represented graphically in Fig.7. For principal bandby SDS-PAGE the Mean and SD values obtained are 98.432 and 1.317 respectively. The same by HZE are 98.476 and 1.415 respectively.The two-tailed P value obtained is equal to 0.3353 and by conventional criteria this difference is considered to be not statistically significant. t=0.9832 at 95% confidence interval and standard error of difference observed is 0.045.

The mean and SD for other bands by SDS-PAGE are 1.560 & 1.325 respectively. The same by HZE are 1.536 and 1.403 respectively. Percentage of other bands observed by both methods for 25 samples is represented graphically in Fig.8. The two-tailed P value for other bands equals to 0.6032, this difference is also considered to be not statistically significant. t= 0.5267 at 95% confidence interval and the standard error of difference obtained is 0.046.

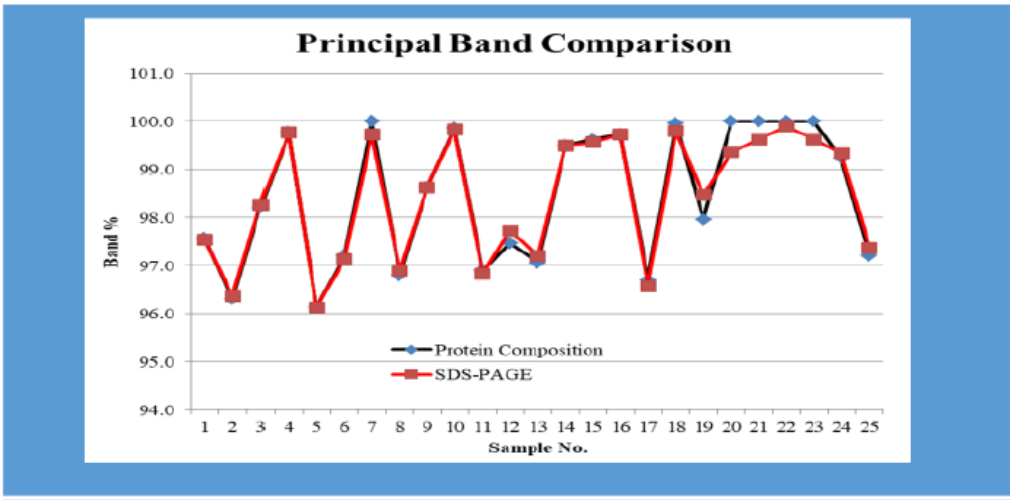


Fig.7: Comparison of value (%) obtained for Principal Band by SDS-PAGE & HZE.

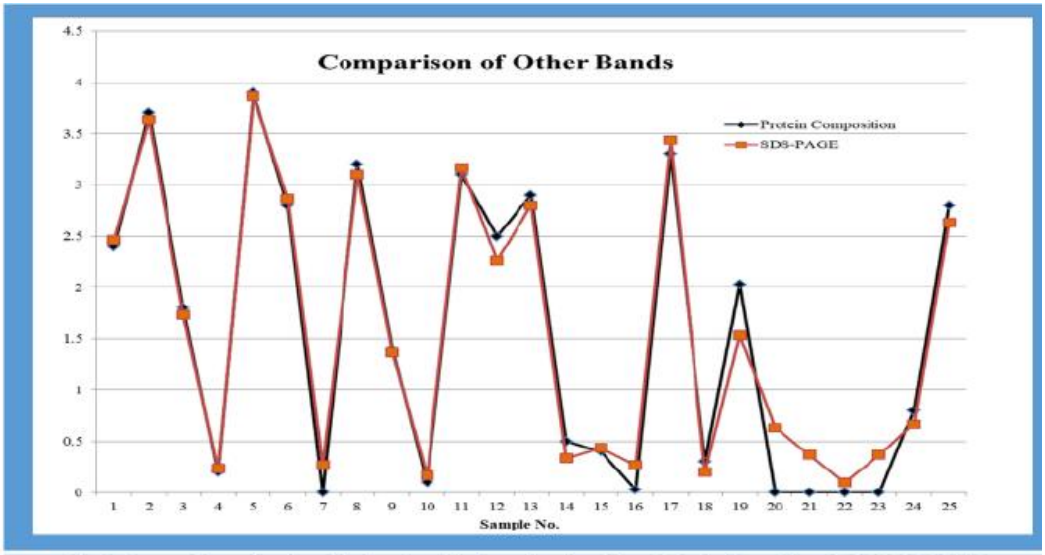
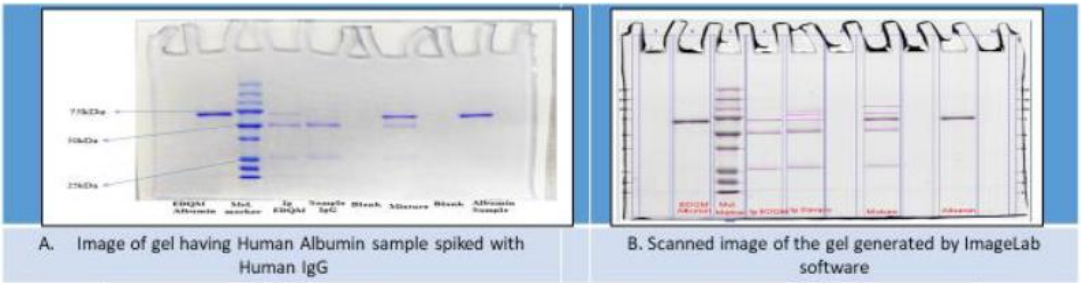


Fig.8: Comparison of value (%) obtained for bands other than Principal Band by SDS-PAGE & HZE.

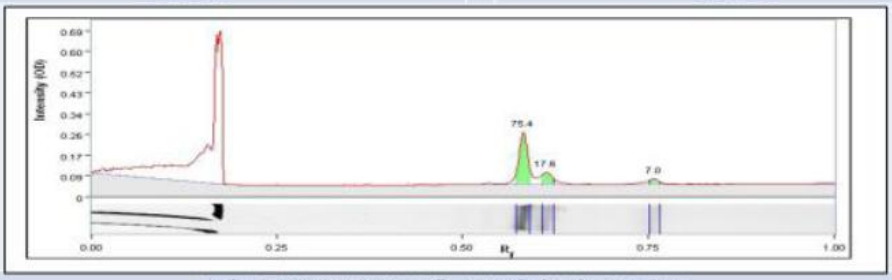
IV. Resolution:

The resolving power of the gel (5-10%) was assessed by running a human albumin sample spiked with human IgG along with EDQM reference standards for human albumin and human immunoglobulin. The results observed with images of gel and analysis are given in Fig.9. The gel resolved the 150kDa immunoglobulin in two bands; first band with approx. 55kDa molecular weight was related to heavy chains of human IgG. The other band represented the light chain of human IgG with approx. 25kDa molecular weights. The percentage of Principal band of human albumin was reduced to 75.4% and human IgG was detected as impurity with 17.6% and 7.0% (a total of 24.6%)



A. Image of gel having Human Albumin sample spiked with Human IgG

B. Scanned image of the gel generated by ImageLab software



Analysis : Lane – 7 - Human Albumin spiked with Human IgG.

Band No.	Mol. Wt. (kDa)	Relative Front	Volume (OD)	Band%
1	68.9	0.581	427.66	75.4
2	56.7	0.612	99.76	17.6
3	35.0	0.757	39.79	7.0

Fig.9 Resolution

V. Discussion

SDS-PAGE technique is robust and dependable for determination of molecular weight of a sample and also for determination of purity or impurities. The results obtained to determine the purity or impurities in Human Albumin samples were statistically analyzed by GraphPad Prism software. The t- score obtained for the principal band was 0.98 and for other bands 0.53. Both the values were < 1.711 at 95% confidence interval. Hence, it was statistically proven that "Protein Composition by Horizontal Zone Electrophoresis using cellulose acetate strips can be replaced by SDS-PAGE technique to determine the purity or impurities in Human Albumin samples.

Moreover, as we are aware SDS-PAGE holds several advantages over Horizontal Zone Electrophoresis.SDS-PAGE is robust, cost effective and reagents are easily available. The technique is ease to perform and one gel can accommodate multiple samples at a time which saves time and labour of the analyst. Cellulose acetate membranes are costly and can accommodate only one sample per strip/membrane. SDS-PAGE is more sensitive than Horizontal Zone Electrophoresis, because it separates proteins on the basis of their molecular weights rather than their charges; additionally on comparison it was observed that it can detect even minor impurities in samples where HZE has shown zero impurity. In adition, Protein Composition by HZE involves using of Amido Black dye for staining protein bands on the cellulose acetate strips and SDS-PAGE practices Coomassie Blue or Silver staining for the detection of protein bands on the polyacrylamide gels.The detection limit of amido black is >100ng and Coomassie Blue can detect even 100ng protein band. The detection sensitivity of amido black is lower than that of Coomassie Blue. To make more sensitive technique for detection, silver staining of SDS-PAGE can also be done to detect even 0.1ng protein present in the gel[22]. Tris buffer was used in SDS-PAGE which is easily available for purchase to testing laboratories. Prior approval is necessary for purchase, usage and storage of Barbital buffer (narcotic drug) and if inhaled, it can cause severe harm to the analyst.

VI. Summary And Conclusion

Protein Composition determined by Horizontal Zone Electrophoresis (HZE) is defined in Pharmacopoeia (IP/EP/BP) for estimation of purity or impurities in human albumin samples[8-10]. Monograph refers the testing on cellulose acetate strips/ agarose gel,using barbital bufferpH 8.6,which is a classic example of horizontal zone electrophoresis. In the "electropherogram obtained with the test solution on cellulose acetate or on agarose gel, not more than 5% of the protein has a mobility different from that of the principal band" is the criteria for quality control evaluation.SDS-PAGE method using calibrated densitometer for analysis can bean alternatetoHZE as it facilitates quantification ofPrincipal and other bands in human albumin samples. The calibrated molecular marker was also run along with reference standard (EDQM, France) and Human Albumin samples, which gives an edge to the technique for the estimation of purity index and molecular weight of the

albumin samples. Bands similar to International Reference Standard- EDQM, France can also be used to validate the results obtained from the experiment. SDS-PAGE is already being used as an effective tool for identification purpose of various Biologicals, such as Human Coagulation Factor VIII (rDNA) [17,21].In conclusion, SDS-PAGE was found more reliable, robust and sensitive technique for quality control evaluationof human albumin samples in comparison to the already existing Protein Composition by HZE. Hence, SDS-PAGE with calibrated densitometry method can be added as an alternate to existing HZE in Pharmacopoeia for determination of purity or impurities incommercially available human albumin solutions prescribed for various therapeutic treatments.

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