

Purification of Glycated Hemoglobin from Hemolysate of Blood Samples and Its Application in Immunoassay.

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Abstract: This study was embarked on, to produce working standards for HbA1c ELISA assay, using locally accessible materials. A pool of blood samples with high levels of HbA1c were used to make hemolysate. Estimation of Hemoglobin level and % HbA1c was done, by Bradford assay and Biorad D-10 respectively, prior to loading on Sephadex G-100 column. Peaks eluted for different hemoglobin variants were purified and confirmed by SDS PAGE. The range of working standards for in-house ELISA assay for HbA1c was made from purified fractions of HbA1c. The concentration of Hemoglobin and HbA1c were 3.3g/ml and 20% prior to loading on Sephadex G-100 column and was abridged to 3.0g/ml and 18% respectively as assessed by Bradford assay. The molecular weights of HbA1a, HbA1b, HbA1c and HbA0 peaks, eluted from the Sephadex G-100 column, were confirmed by SDS PAGE as 66KD-68KD. The in house working standards prepared from HbA1c purified fraction were in the range of 2% - 18% and confirmed with the commercial HbA1c ELISA kit. The standard points, prepared for HbA1c ELISA assay, can be used at local clinical setups for routine screening and management of Diabetes Mellitus to make the test economical.

Keywords: Diabetes mellitus, Glycated Hemoglobin, HbA1c ELISA.

I. Introduction

Diabetes mellitus is a metabolic disorder categorized by high blood glucose, if not controlled can lead to a number of chronic complications as nephropathy, retinopathy, neuropathy, diabetic foot and cardiovascular diseases [1]. The ratio of diabetes is increasing globally at a high rate with an increase in annual expenditure. The estimated annual expenditure on diabetes in 2007 was about “\$232 billion” and it is expected to be “\$305” billion till 2025 [2]. The pre-diagnostic period of Diabetes mellitus lasts up to seven years before establishing full disease based on symptoms such as polyuria, blurred vision and increased thirst. Thus, screening of diabetes in pre-diagnostic phase could decrease the risk of morbidities [3]. Several tests are available for diagnosis and prognosis of diabetes, such as Fasting plasma glucose (FPG) level, oral glucose tolerance test (OGTT) and estimation of HbA1c. HbA1c test is not only used for screening, but also helps clinicians to understand the pathway of glycation of Hemoglobin (Hb) in RBCs during a four month period [4].

Glucose molecule binds to β -chain of Hb after a non-enzymatic reaction and results in Glycated Hb i.e. HbA1c. The higher the glucose level of the blood higher will be the rate of glycation of Hb [5]. A one percent change in Hb results from 2mmol/L changes in mean blood glucose level. HbA1c levels 5% or less is considered normal while $\geq 5\%$ up to 6.0% at risk of developing Diabetes Mellitus, 6.0% to 6.5% as diabetes under control and $\geq 6.5\%$ as uncontrolled Diabetes Mellitus [6]. It is recommended that diabetics should keep their HbA1c level $\leq 6\%$, i.e. 42 mmol/L without an excessive decrease in glucose level below normal range, which could be risky to the patient and can lead to impediments [7].

Due to availability of a large number of methods for determination of HbA1c and the existence of various variants of hemoglobin that interfere with HbA1c, standardization of methods is essential [8]. Available methods for determination of HbA1c are Ion exchange chromatography, Boronate-affinity chromatography, HPLC, Electrophoresis and Immunoassay [9].

An enzyme immunoassay has been developed in Pakistan using the imported HbA1c standard points. This study was carried out to prepare HbA1c standards, using locally available raw materials to save the foreign exchange on import of standard points and to bring the test within means for screening and management of Diabetes Mellitus.

II. Materials And Methods

Sephadex G-100, TRISMA base, TEMED and Bromophenol blue were procured from Sigma, Protein molecular weight marker and Glycine from Fermentas, APS, SDS and Glycerol from ICN. 2-Mercaptoethanol from Fluka. Acrylamide and Bisacrylamide from Merck. All other reagents were of analytical grade and purchased from Merck and Riedel-De-Haen.

A total of 60 blood samples of ambrosias diabetic patients without identification, ranging from 8.0%-16.0% as read on an automated system from Biorad D-10, were collected from Biochemistry Laboratory of Shaikh Zayed Hospital as professional help. All samples were pooled and centrifuged at 3000rpm for 10 minutes. Plasma was separated using a glass Pasteur pipette. Red blood corpuscles were lysed at 37°C with 50mM phosphate buffer (pH 7.4), equal to the volume of plasma removed.

Erythrocytes were again centrifuged for 20 minutes at 3000 rpm at room temperature to remove labile glucose adducts. Supernatant (15ml) was decanted and water equal to the removed supernatant and 0.4 volumes of CCl₄ was added and mixed on a roller mixer for 20 minutes at room temperature to separate lipids and lipid-soluble products. Finally, Erythrocytes were centrifuged at 3000rpm for 15 minutes at room temperature to remove cell debris and hemolysate was stored at -40°C for further use.

Total hemoglobin concentration of hemolysate was estimated using the Bradford assay. Different concentrations of BSA were prepared and diluted at 1:10 in deionized water. To each concentration, 5ml Bradford reagent was added and incubated for 10 mins at 37°C and read at 595nm. The optical density of each concentration was plotted to generate the standard curve. Stock hemolysate was diluted at 1:100 in 50 mM phosphate buffer to prepare the working stock. The working stock was further serially diluted to cover the range of interest in the linear region of the graph. Concentration was then estimated from the absorbance at 595nm.

To purify HbA1c from other Hb variants, the Sephadex G-100 column was washed and equilibrated with 25mM phosphate buffer (pH 6.6) overnight at a flow rate of 20ml/hr. To elute the fractions, 50mM phosphate buffer (pH 6.6) with a linear gradient of NaCl (0M-1.0M) was used. Total 56 fractions of 3ml each were collected, absorbance was read spectrophotometrically on 415nm, 540nm and 670nm respectively, and generated peaks for five Hb variants.

For further confirmation of Hb variants SDS PAGE was run on samples of each generated peak and bands were produced using the highest concentration of the respective Hb variant.

From HbA1c concentration eluted from chromatography column, an 18% stock standard was prepared and mixed with already available 2% HbA1c standard to generate a series of working standards to be used in in-house HbA1c ELISA assay.

2.1 Assay Protocol:

Two Anti-HbA1c antibody coated wells of microtitre plate, 10µl of working standard points ranging from 2%-18% were added in duplicate. For optimum binding of HbA1c to its antibody Microtitre plate was incubated for an hour at room temperature. The supernatant was discarded and the wells were washed for five times with PBSX (25mM PBS + 0.1% Triton X) to remove the unbound fraction followed by 100µl of biotinylated antiHbA1c at 1:10K dilution in each well and incubated for an hour to allow optimum binding. The unbound fractions were discarded and washed five times with PBSX followed by 100µl of streptavidin-HRP at 1:5K and incubated for half an hour. The unbound streptavidin-HRP was discarded and the wells were washed 4 times. TMB substrate was added to each well and incubated for 20 mins for color development. The reaction was stopped by adding 100µl of stop solution (1M H₂SO₄) and wells were read at 450nm/620nm within 30mins. For precision of assay micro/multi-channel precalibrated pipettes from Gilson were used and incubation was carried out at room temperature throughout the assay.

III. Results And Discussion

3.1 Bradford assay

For the estimation of Hb concentration of hemolysate, BSA stock solution was diluted serially and a linear graph was plotted against the absorbance at 595nm (Fig. 1). Hemolysate was diluted at 1:100 in PO₄ buffer.

Absorbance of BSA dilutions was read at 595nm and Hb concentration in hemolysate was calculated using the equation. The Hb concentration was found to be 3.3g/ml.

3.2 Gel Filtration Chromatography

Further purification of HbA1c from Hb variants was carried out using gel filtration chromatography technique. A dilution of 1:100 of hemolysate in phosphate buffer was applied to the Sephadex G-100 column. Hemolysate was allowed to adsorb onto the gel and fractions were eluted with 50mM phosphate buffer with a linear gradient of NaCl from 0-1.0M concentration, 56 fractions of 3ml each were eluted and read at 415nm, 540nm & 670nm. The absorbance at 540nm was found specific for Hb in five generated peaks, the first peak was predicted for HbA1a₁ second peak for HbA1a₂ and third, fourth and fifth peaks were for HbA1b, HbA1c and HbA^o respectively as shown in Fig 1.

3.3 SDS-PAGE

To confirm the Hb variants in fractions purified on the Sephadex G-100 column, samples of peak fractions were applied on SDS-PAGE. The protein bands obtained were of molecular weight in a range of 66-68KD (Fig 2), confirming the presence of Hb variants.

3.4 Preparation of working standard

The estimated concentration of High standard of HbA1c prepared from hemolysate was 18% (Biorad D-10), whereas lower standard of 2% HbA1c was taken from NHRC laboratory. These two standards were used and a stock of five HbA1c standards of varying concentration, i.e. 2%, 4.5%, 9%, 13.5% & 18%, was set.

The working standards using stock standards were prepared with (SIPAC) buffer, 0.1% triton at 1:10 dilution.

3.5 Application of standard points in ELISA

Anti HbA1c coated wells were used to test the prepared HbA1c working standards (2%, 4.5%, 9%, 13.5% and 18%). The absorbance read at 450/620nm was plotted against the concentration to generate a standard curve (Fig 3). The concentration of quality control, pools and unknown samples were read from the standard curve against the absorbance.

IV. Discussion

Estimation of HbA1c level is a standard test for screening and management of Diabetes Mellitus. In Pakistan an in-house HbA1c ELISA assay has been developed, the working standards for which were being imported. To reduce the cost, the current study was undertaken to develop working standards from blood samples of diabetic patients having high HbA1c levels. Using the Peterson method [10] hemolysate was prepared from blood samples of diabetic patients and was found to have a hemoglobin concentration of 3.3g/ml as estimated by Bradford method and an HbA1c concentration of 20% as estimated by Biorad D-10. A linear gradient of NaCl 0M-1.0M in 50mM sodium phosphate buffer was used to elute the peaks for Hb variants, peaks eluted were similar to the peaks eluted by Moussa in 2007, i.e. highest peak was of HbA1a, followed by HbA1b, HbA1c and HbA0, about 70-80% of these fractions were of HbA1_{a+b+0} and only 20-30% was of HbA1c [11]. Huisman & Dozy, reported using fast flow chromatographic columns, purification of HbA1c and other fast moving variants of Hemoglobin, of which 7% was HbA and 70-80% was HbA1c [12]. In the current study gel filtration chromatography, has generated contradictory results, i.e. 70-80% of the fractions were for HbA1_{a+b+0} and remaining were of HbA1c. Moreover, after gel filtration, as a result of purification, HbA1c was reduced to 18% and Hb to 3.0g/ml. This difference in results could be due to variation in NaCl concentration or pH change. Ellis in his study has also reported, the change in pH and NaCl concentration could result in incomplete isolation of HbA1c [13].

Furthermore, the results of eluted peak fractions on 10% SDS-PAGE in this study, generated bands of 66kD-68kD, specific for Hb variants as shown in figure 2. The increase in molecular weight of the variants than of normal Hb is due to glycation of the β -chain of hemoglobin. In the current study, it was confirmed that the intact band of 68kD in 4th well is specific for HbA1c fraction (Fig 2) as it was at the same position as the band generated by Thorman for HbA1c, in his experiments after isolation by Capillary Electrophoresis and SDS [14].

This method can be opted at clinical setups without the need of any special control conditions needed by other methods for purification of HbA1c, Kaplan et al., reported, the slight temperature variation could interfere with purification of HbA1c using minicolumn, their study showed the best results obtained are in a defined range of temperature, i.e., 22°C-24°C [15]. Another study by them has shown, if the temperature is controlled throughout the experimental settings by the use of Calibrators, the impact can be minimized [16]. Such an impact of temperature, on purification of HbA1c, was not found in our case by gel filtration chromatography as the experiments were performed at room temperature.

The results obtained by immunodetection were precise as confirmed by the linearity of the standard curve. The straight line obtained is a reflection of no interference by other Hb Variants, hence accuracy for detection of HbA1c value by this method [17]. Whereas in their study Agilli et al. in 2013, reported that HbA1c measurement using Ion exchange chromatography and HPLC gives falsely elevated levels i.e. about 11% higher than the original value due to interference of HbH. Similar results were also observed by Nadzimah, Thevarajah and Chew in 2010, who reported interference of Hb F, Hb S, Hb C and Hb D variants in the estimation of HbA1c levels [18].

The in-house ELISA assay is also a fast approach as compare to other methods, like HPLC, it takes about 150 mins to estimate 45 samples in duplicate, another advantage of Immunoassay is that it is not only faster, but has also become economical because of producing working standards in the laboratory from the pool of patient samples with high HbA1c levels, which are discarded after the estimation of required tests. Ozcelik et

al.,in 2010, compared three methods for detection of HbA1c andreported, Immuno-detectionas a fast method as it takes 45mins as compared to 385mins by HPLC [19].The in-house ELISA assay is not only useful for screening and management of Diabetes Mellitus by determining HbA1c, but also for research at economical cost.

V. Conclusion

This study was embarked on to make standard points, to be used with an in house ELISA assay, from the scrap and has completed with a productive result. The standard points prepared are in a range of 2% -18% HbA1c value and can be utilized efficiently at local clinical setups to have an insight into the diagnosis and prognosis of Diabetics. This simple method of using diabetic's blood with high concentration of HbA1c, that is usually discarded after glucose estimation, will reduce the cost of importing the standards and will surely help many of the laboratories especially in developing countries.

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Figures

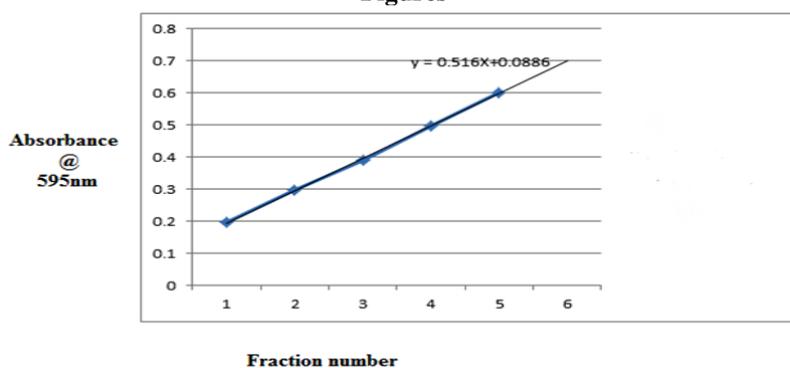


Figure 1. Standard curve of BSA at 595nm.

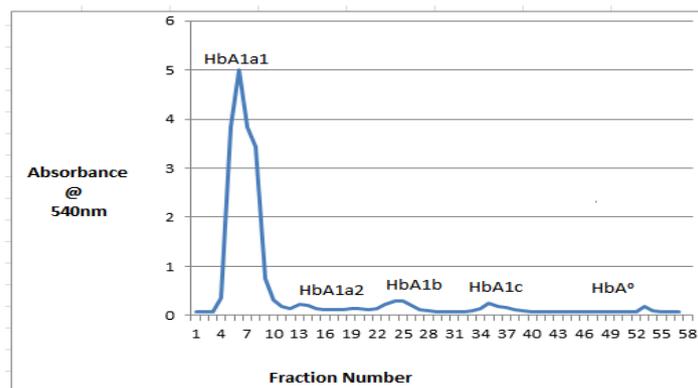


Figure 2: Peaks of Hb variants at 540nm. Peak 1 (HbA1a1), Peak 2 (HbA1a2), Peak 3 (HbA1b), Peak 4 (HbA1c) and Peak 5 (HbA0).

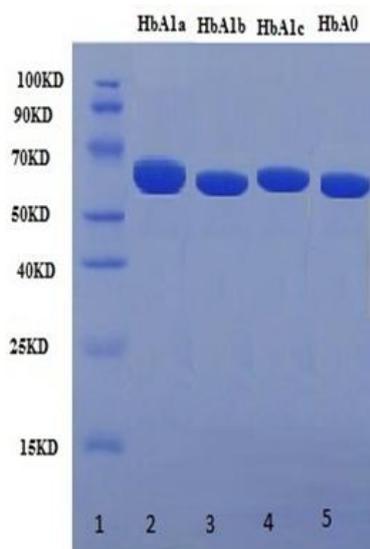


Figure 3: Bands of Hemoglobin variants obtained from SDS-PAGE. Well 1 protein ladder, Well 2, 3, 4 and 5 HbA1a1, HbA1b, HbA1c and HbA0 respectively. Molecular weight for Hb variants range between 64KD to 68KD.

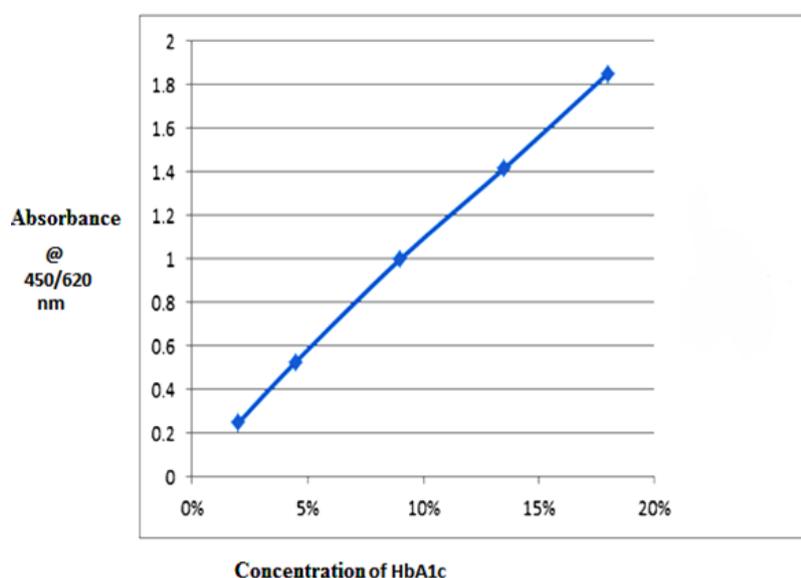


Figure 4. Standard curve for working standard points in HbA1c in-house ELISA assay