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Refractive index of blood is a potential qualitative indicator of hemoglobin disorder in humans

Pushpanjali Dasauni[1], Manoranjan Mahapatra[2], Renu Saxena[2] and Suman Kundu[1]*

[1]*Department of Biochemistry, University of Delhi South Campus, New Delhi-110021, India.*
[2]*Department of Haematology, All India Institute of Medical Sciences, Ansari Nagar, New Delhi-110029, India.*

***Corresponding author**: Suman Kundu. Tel.: +91-1124112081.
E-mail:suman.kundu@south.du.ac.in;

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Abstract

Hemoglobin disorders are inherited genetic diseases and a global health burden. The routine diagnostic tools like iso-electric focusing and high performance liquid chromatography based methods are successful in many cases but sometimes yield ambiguous results and are complex, labour-intensive and time consuming. There is thus pressing need of simple molecular tools for identification of hemoglobin disorders. As such, the aim of the present study was to explore a simpler optical property of blood viz. refractive index to differentiate between the blood sample of healthy individuals and those of individuals with hemoglobin disorder. Blood samples were collected from 43 healthy and 63 hemoglobin disorder patients and the refractive index was measured at different processing stages of blood (whole blood, plasma, hemolysate and pure hemoglobin) by using refractometer. The total protein concentration in whole blood was estimated using refractive index in relation to Bradford's assay and spectrophotometric estimation. The refractive index was found to be directly proportional to total protein concentration. The refractive index values for diseased samples were found to be significantly lower than the healthy controls for whole blood samples, probably due to lower protein content in the former. The change in refractive index values between healthy controls and diseased samples were however not significant in plasma, hemolysate and pure hemoglobin samples probably due to inconsistent dilution during sample processing. Whole blood is thus the material of choice to differentiate between the healthy and the diseased samples using refractive index. Refractive index of blood could serve as a potential tool for qualitative diagnostics of hemoglobin disorders in near future.

Keywords:

Hemoglobin disorders; refractive index; whole blood; hemolysate, diagnostics; hemoglobin variants; Hemoglobind.

Introduction

Hemoglobin (Hb) disorders are the most common genetic disorders throughout the world. About 7% of the world population is affected with Hb disorders (Balgir, 2000; Das et al., 2016). In India, HbS, HbE, HbD-Punjab, HbE-βThalassemia and β-Thalassemia are the most frequently occurring Hb disorders (Balgir, 2000). These diseases are fatal when untreated and are a major cause of morbidity and mortality in children below the age of 5 years (Scott et al., 2014). These disorders are caused due to any alteration in the Hb molecule. Defects can be qualitative which are caused due to deletion/addition/substitution of codons in the genes of hemoglobin chains, giving rise to hemoglobin variants like HbS, HbE etc. or quantitative which are caused due to decreased production of any of the globin chain e.g. thalassemias (Forget and Bunn, 2013).

Sickle cell disease (HbS) is caused due to mutation in β-chain at the 6th position where glutamic acid is substituted with valine (Ilesanmi, 2010). HbE disease is caused due to mutation in the β-chain at the 26th position where glutamic acid is substituted with lysine (Fucharoen and Weatherall, 2012). HbD-Punjab disease is caused due to mutation in the β-chain at the 121st position where glutamic acid is substituted with glutamine (Torres et al., 2015). The symptoms associated with these disorders commonly include anemia, splenomegaly, poor growth, problems related with heart, liver and bones, pain syndromes and swelling of hands and legs. Treatment available is mostly symptomatic; folic acid supplementation, hydroxyurea (Youssry et al., 2017) and L-glutamine (Aschenbrenner, 2017) are the only FDA approved drugs available which reduce the symptoms in sickle cell disease. In severe cases blood transfusion and iron chelation therapy can be provided. They are majorly distributed in Africa, Asia, Mediterranean basin and the Middle-east countries (Kohne, 2011; Weatherall, 2008). Their prevention, control and treatment totally rely on the diagnosis of the disease. The diagnostic tools that are most commonly used are iso-electric focussing (IEF) and high performance liquid chromatography (HPLC) based methods which are successful in many cases but sometimes offer ambiguous results due to co-migration and co-elution of variants (Wajcman and Moradkhani, 2011). WHO in 2002, emphasized on introduction of simple molecular technology for their diagnosis and control (WHO, 2002). Optical properties of blood could be utilized for such purposes since they need extremely small quantities of blood and provide fast, reliable and inexpensive measurements.

Refractive index is an in-expensive, non-invasive method which can estimate the concentration of samples on the basis of light scattering properties. Optical properties of blood are important for diagnostics, treatment and management of disease. Barer in 1957 was the first to define the refractive index of hemoglobin at 589 nm (Barer, 1957) but since then this has been a largely un-explored diagnostic tool for hemoglobinopathies. Earlier studies have shown its potential to measure the refractive index of blood as an alternative tool in the identification of diabetes mellitus (Mazarevica, 2002). Moreover, optical parameters like refractive index and absorption can be utilized for the quality control of blood samples for transfusion purposes like estimation of the amount of glycerol in blood (Wong et al., 2009) and for evaluating hemoglobin concentration (Fabry and Old, 1998). Park and colleagues demonstrated that the refractive index act as an indicator for cell morphology and pathological changes in *P. falciparum* infected RBCs (Park et al., 2008). In blood, plasma protein concentration was determined by refractometry (Weeth and Speth, 1968), which seems to be a sensitive indicator of blood concentration and dilution. The optical parameters of blood is contributed by interaction between plasma and erythrocytes (RBCs) (Yaroslavsky et al., 2002), which is 99% for RBCs due to the presence of hemoglobin (Meinke et al., 2005). Douplik and group monitored the refractive index of oxy and deoxy hemoglobin for nine wavelengths in range from 400-700 nm (Zhernovaya et al., 2011). They found that for the different forms (like oxy, deoxy, met) of Hb, refractive index remains same and it changes with temperature fluctuations majorly due to change in refractive index of water. Thus refractive index could be a robust indicator of Hb concentration in blood, which could then serve as a fast and reliable diagnostic tool for Hb disorders.

To our knowledge, no literature is available where refractive index was used to differentiate between the healthy and the Hb disorder blood samples. It is thus important to explore refractive index as a potential tool to study hemoglobin disorders. The aim of our study was to estimate the hemoglobin concentration by measuring refractive index and to compare refractive index of blood samples of healthy controls and hemoglobin disorder patients, where Hb disorder was confirmed first by regular screening. Results indicate that such distinction between the blood of healthy individual and those with hemoglobin disorders may be possible.

Materials and Methods

Materials - Hemoglobind matrix was procured from Biotech Support Group, LLC (New Jersey, USA). This matrix binds hemoglobin specifically from a mixture of other biomolecules.

Ethical approval and blood sample collection - Ethics approval for the study was obtained from University of Delhi South Campus (UDSC) (3/IEC/SK/Biochem/UDSC/18.2.2014) and All India Institute of Medical Sciences (AIIMS) (IEC/OP-4/01.05.2014). Written consents were obtained from the volunteers before blood collection. Blood samples from healthy individuals were collected at UDSC and those from patients suffering from Hb disorders at AIIMS. The patients were confirmed to have Hb disorders based on regular screening and haematological parameters. Venous blood samples (3 ml) were collected in EDTA vials and kept at 4°C till the processing step. After processing, the samples were kept at -20°C till further analysis. Total 43 healthy blood samples and 63 diseased samples i.e. 14 with HbS, 15 with HbE, 4 with HbD-Punjab, 4 with HbE-β-thalassemia and 26 with β-thalassemia mutations, were collected.

Sample processing - The blood samples were processed as described below. Whole blood samples were centrifuged at 400xg for 10 min to separate plasma, which was stored in separate vials. The red blood cells (RBCs) were washed thrice in 0.9% saline to remove buffy coat and to pack RBCs. Packed RBCs were added with double volume of distilled water and centrifuged at 20,000xg for 30mins. This pellets all the cell stroma and provides hemolysate as supernatant (Liu et al., 2003). Pure hemoglobin was isolated from hemolysate by using Hemoglobind reagent as several groups have used this resin for removal of hemoglobin from composite samples (Saraswathi et al., 1999; Bhargava et al., 2016; Laing et al., 2017; Carvalho et al., 2017). The hemolysate was added to an equal volume of Hemoglobind resin and mixed properly and centrifuged at 400xg for 2min to pellet down the hemoglobin bound to Hemoglobind reagent while unbound hemoglobin was washed away. The hemoglobin bound to the matrix was eluted by increasing the pH of the buffer using 100mM tris borate, pH 9.

Bradford assay and spectrophotometric (Nanodrop) estimation of protein content - The protein concentration in whole blood was estimated using Bradford's method and spectrophotometric method using Nanodrop 2000/2000c (Thermo Fisher Scientific,

Wilmington, USA). Whole blood sample was incubated in Bradford's dye for 10 min and then the absorbance were measured at 595 nm and protein concentrations were calculated using standard curve obtained by using known concentration of BSA. In Nanodrop method, the sample was diluted (20 fold) to measure absorbance of whole blood sample at 280 nm. Similarly, absorbance was recorded without dilution for the plasma, hemolysate and pure Hb samples. Nanodrop 2000/2000c software on Nanodrop instrument was used to estimate the total protein concentration. Further the samples were run on 15% SDS-PAGE gel to validate the accuracy of protein concentration as calculated by Bradford's assay and spectrophotometric measurements by Nanodrop.

Refractive index measurements - The refractive index of all the healthy controls and diseased blood samples were determined using refractometer **(**AR4, A. KRÜSS Optronics GmbH, Hamburg, Germany). All the experiments were done in triplicate and measurements were done for samples at all stages of processing i.e. whole blood, plasma, hemolysate and isolated Hb. Different ratio of 0.9% saline (100-0μl) and blood samples (0-100μl) was mixed to measure their refractive index with dilution.

Instrumentation - The refractive index of whole blood, plasma, hemolysate and pure Hb samples were determined by refractometer. The sample was sandwiched to a thin layer between the illuminating prism and the refractive prism. From the eyepiece, the crosshair section was adjusted by a horizontal line to half light and half dark region and the reading was recorded to get the refractive index.

Statistical analysis - Refractive index measurements were performed in triplicates and data is represented as mean ± standard deviation. For the study, the control and the diseased samples were analyzed in four processing stages of blood samples. The statistical difference between the four groups were determined by using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison test. P Value of ≤ 0.05 was considered statistically significant. GraphPad Prism (version 5.01; GraphPad Software, Inc., CA, USA) was used for performing all the statistical calculations and creating all the graphs.

Results

IEF and HPLC based methods have been in use to diagnose Hb disorders at the protein level, but they are often laborious, time consuming and suffer from other

disadvantages like co-migration and co-elution of different mutated Hb chains creating ambiguity in detection. Other methods like mass spectrometry and FTIR spectroscopy are yet to be clinically used and often rely on estimation of Hb concentration or total protein concentration in whole blood. Literature search (as described in Introduction) and routine use of refractive index measurements in the laboratory for measuring concentration of biological reagents like guanidium chloride and urea led us to the hypothesis that the simple optical tool of refractometry may be extended to the estimation of protein content in blood and thus in differentiating blood of patients suffering from Hb disorders from the blood of healthy individuals. To verify this hypothesis, protein content in blood was diluted to multiple dilutions and the refractive index measured for each dilution. For comparison, the protein content at each dilution was also estimated by standard, universal methods like Bradford's assay and spectrophotometric measurement of absorbance at 280 nm using a widely popular spectrophotometer called Nanodrop that utilizes a drop of protein solution for measurements.

In the first instance, different ratio of 0.9% saline (100-0 μl) and whole blood (0-100μl) were used to make the dilution (**Table 1**). It was found that protein concentration increases progressively as higher blood volume was used for both the Bradford and Nanodrop based methods (**Table 1**). However, it was observed that the protein concentration estimated by Bradford assay was inconsistent with the volume of whole blood used (**Table 1**). The Bradford assay suffered from inconsistency probably due to interfering agents in the processing buffer and hence not advisable for use for concentration determination for biochemical and diagnostic purposes. The refractive index, like Nanodrop estimation, also showed consistent increase (**Table 1**). The protein concentration thus estimated was validated by intensity of bands on SDS-PAGE against known concentration of protein (data not shown). The plot of refractive index against total protein concentration in whole blood showed remarkable consistency and linearity (**Figure 1**), indicating that refractive index is a reliable measure of protein concentration or blood density.

Since refractive index varied consistently with blood content concentration, and it is expected that blood from individuals suffering from hemoglobin disorder (diseased blood) may have differences in concentration compared to blood from healthy individuals (control blood), refractive index was used to investigate diseased blood. In addition, blood of patients suffering from Hb disorder could have differences in viscosity or other physical properties that may result in differences in refractive index. We thus measured the refractive index of

control and diseased blood samples to see whether we can differentiate between them. The refractive index values in different processing stages of blood i.e. whole blood, plasma, hemolysate and pure Hb were measured in triplicate using blood from each individual separately. The refractive index values from individual to individual showed remarkable consistency (data not shown). Finally, the refractive index value of each individual were averaged and reported. The average refractive index of whole blood samples in controls and diseased blood are shown in **Table 2**. The average refractive index value of whole blood samples in 41 healthy control samples is 1.374 ± 0.0061 (mean ± SD) (**Table 2**). In comparison, the refractive indices of diseased whole blood samples are illustrated in **Table2.** Refractive index in 10 HbS samples is 1.364 ± 0.0039, in 15 HbE samples is 1.365 ±0.0042, in 3 HbD-Punjab samples is 1.367 ± 0.0032, in 3 HbE-β-thalassemia samples is1.362 ± 0.0056 and in 24 β-thalassemia samples is 1.364 ± 0.0044 (**Table 2**). It is evident that the refractive index values of whole blood in diseased samples are significantly lower, as compared to healthy controls (when $P<0.05$) which suggest low concentrations of hemoglobin in these samples (**Fig. 2A**). The common symptom of all Hb disorders is anemia and such differences may be expected.

The average refractive index in plasma samples was found to be 1.350 ± 0.0029 in 43 healthy control samples (**Table 2**), which is significantly lower than whole blood samples. In diseased plasma samples the average refractive index was found to be 1.351 ± 0.0019 in 11 HbS samples, 1.351 ± 0.0024 in 12 HbE, 1.350 ± 0.0015 in 4 HbD samples, 1.352 ± 0.0028 in 4 HbE-β-thalassemia samples and 1.351 ± 0.0026 in 26 β-thalassemia samples (**Table 2**). The refractive index values of plasma in diseased samples are slightly higher (insignificant) as compared to healthy controls (**Fig. 2B**). This may be due to higher fragility in the diseased RBCs which results in leakage of Hb in the plasma samples, since the plasma samples having slightly higher refractive index values were red in color. In summary, the diseased plasma samples do not show differences in refractive index from healthy controls.

The average refractive index in hemolysate samples was found to be 1.351 ± 0.0169 in 40 healthy control samples (**Table 2**). In diseased hemolysate samples the average refractive index was found to be 1.340 ± 0.0028 in 12 HbS samples, 1.340 ± 0.0028 in 12 HbE samples, 1.342 ± 0.0021 in 2 HbD samples, 1.339 ± 0.0021 in 2 HbE-β-thalassemia samples and 1.342 ± 0.0016 in 25 β-thalassemia samples (**Table 2**). When $P<0.05$, the

refractive index values of hemolysate shows significant differences between control and diseased samples except for HbD samples which shows insignificant difference from control samples (**Fig. 2C**). This suggests decreased concentration of Hb in diseased samples but these differences are less from the whole blood samples because during sample processing successive dilutions take place. For HbD, only 4 samples were obtained by us and once higher numbers of samples are analyzed, statistical difference may show up.

The average refractive index in pure Hb samples was found to be 1.337 ± 0.0022 in 20 healthy control samples (**Table 2**). In diseased pure Hb samples the average refractive index was found to be 1.335 ± 0.0009 in 15 HbS samples, 1.335 ± 0.0003 in 11 HbE samples, 1.355 ± 0.0 in 2 HbD samples, 1.355 ± 0.0 in 3 HbE-β-thalassemia samples and 1.355 ± 0.0 in 6 β-thalassemia samples (**Table 2**). When $P<0.05$, the refractive index values of pure Hb between control and HbS shows significant differences but with other diseased sample shows insignificant differences (**Fig. 2D**). The low differences between controls and diseased blood samples indicate that these samples may not be the right ones for diagnostic purposes.

Discussion

Our study shows that refractive index is a direct measure of protein concentration in blood samples and the relationship between protein concentration and refractive index was found to be linear (**Fig. 1**). We find the refractive index of pure Hb as 1.335, at 22°C which is in line with the values observed by Zhernovaya in their findings at 20°C (Zhernovaya, 2011). This suggests that refractive index is directly proportional to the hemoglobin concentration in blood. Compared to the traditional ways of measuring protein concentration like Bradford assay, which showed inconsistency, refractive index provided a simple, easy and robust method to estimate the Hb concentration. The Hb concentration is an important marker for anemia which is a common symptom for hemoglobin disorders. Thus anemia may be directly linked to blood disease condition, which can be further investigated in details.

The average refractive index of whole blood samples in healthy control samples as reported in this study was 1.375. The published value is 1.37 as reported by Li's group (Li et al., 2000) and Sardar and Levy (Sardar and Levy, 1998), suggesting that our value is in good agreement with the literature data. In contrast, the refractive index of whole blood samples in diseased blood samples, which was never measured before to the best of our knowledge,

ranges from 1.362-1.367. This lowering can be due to decreased Hb content in the blood of diseased individuals or due to differences in physical properties of diseased hemoglobin (like partial misfolding may cause soluble aggregates which may differ in refractive index).

This study represents the first investigation of refractive index of blood samples from patients suffering from Hb disorders. The refractive index was studied in different processing stages of blood i.e. whole blood, plasma, hemolysate and pure Hb to differentiate between the control and the diseased samples. We found that whole blood samples showed statistically significant differences among the four groups in their RI values between the control and diseased samples.

Plasma proteins consist of 50-60% albumins, 30% globulins and 4% fibrinogens. The normal serum protein level is 6 to 8 g/dl in blood. Albumin makes up 3.5 to 5.0 g/dl, and the remaining concentration of total globulins (Busher, 1990). Fibrinogen makes up 2 g/L to 4 g/L in the plasma (Tennent et al., 2007). We found the refractive index of plasma to be 1.350 at 589 nm, which is same as the R.I. value of plasma (1.350) as reported earlier (Cheng et al., 2002; Meinke et al., 2011) at 400 nm. In healthy cattles also, refractive index of plasma was determined to be 1.350 (Weeth and Speth, 1968). The refractive index of plasma in diseased samples in our study is 1.350-1.352. These slightly high values in diseased samples are probably due to mixing of hemoglobin in plasma. Due to higher fragility of the diseased RBC membranes, Hb get leaked into the plasma resulting in elevated refractive index (Alaarg et al., 2014). The refractive index of plasma samples are not good indicators of diseased state.

The refractive index of hemolysate samples was also measured and the values were 1.348 in control samples and 1.339-1.342 in diseased samples at 22°C so that the difference are not as significant compared to the whole blood samples, which shows statistically significant differences between the healthy controls and the diseased samples. Thus refractive index measurements of plasma, hemolysate and pure hemoglobin should not be utilized to differentiate between the healthy controls and the diseased samples because they show less differences due to successive dilution upon processing, leakage of Hb from RBCs, etc.

Refractive index is capable of differentiating between the control and diseased hemoglobin disorder samples. The whole blood samples showed statistically significant decrease in refractive index in diseased samples as compared to controls. This is in fact a boon for diagnosis of Hb disorder since blood samples will need minimal processing unlike

plasma, hemolysate or isolated Hb. This study will be of advantage in developing countries where resources are limited and anemia is prevalent. Refractive index measurements will help to identify anemia and to identify Hb disorder at least qualitatively. The results are promising and refractive index has a potential for initial screening of hemoglobin disorders. Since refractometer is extremely cheap equipment, the cost of diagnosis will be considerably low.

However, it may not be possible to discriminate some common cause of anemia (like sideropenia) from the less frequent hemoglobin disorders, based exclusively on the evaluation of Hb concentration, without considering other highly informative hemocytometric parameters including RBC, MCV, MCH, MCHC, RDW and Hb fractionation. Also, it may be difficult for refractive index data to be used to discriminate among different and partially overlapping clinical conditions ranging from asymptomatic alpha or beta thalassemia carriers to non-transfusion-dependent thalassemia and Cooley syndrome. Furthermore, some pathological Hb variants are not associated with low Hb values but, when co-inherited with other hemoglobin disorders, could anyhow cause a relevant clinical phenotype. A screening procedure based solely on this approach would undoubtedly underestimate these conditions. However, this cheap, robust and fast method surely could be a part of screening programmes for a preliminary screening and along with other hemocytometric parameters, disease symptoms and outcome, form an unambiguous diagnostic solution for hemoglobin disorders. In essence, this simple optical tool deserves more attention and warrants further investigation as a potential diagnostic tool.

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Authors contribution

SK conceived the study and its design, supervised, analyzed the data and corrected the manuscript. PD procured the samples, performed data collection, data analysis and prepared the first draft of the manuscript. RS and MM provided us with the diseased samples through ethical clearance and provided intellectual input into the study. All authors read and approved the final manuscript.

Conflict of Interests

The authors declare that they have no conflict of interests.

Abbreviations

FDA, Food and Drug Administration; Hb, Hemoglobin; HPLC, High-Performance Liquid Chromatography; IEF, Isoelectric Focussing; RI, refractive index; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; WHO, World Health Organization; RBC, Red blood cells; MCV, Mean corpuscular volume; MCH, Mean corpuscular hemoglobin; MCHC, Mean corpuscular hemoglobin concentration; RDW, Red blood cell distribution width.

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Table 1: Protein (Hb) concentration in different dilutions of whole blood in saline measured by Bradford assay and Nanodrop spectrophotometric method and refractive index.

S. No.	Saline 0.9% NaCl	Whole blood	Protein concentration (mg/ml)byBradford	Protein concentration (mg/ml)byNanodrop spectrophotometer	R.I.
1.	**100 µl**	0 µl	-	-	1.334
2.	**90 µl**	10 µl	12.0	8.4	1.338
3.	**80 µl**	20 µl	36.1	18.2	1.341
4	**70 µl**	30 µl	50.0	27.5	1.345
5.	**60 µl**	40 µl	52.4	33.5	1.348
6	**50 µl**	50 µl	58.9	53.8	1.351
7.	**40 µl**	60 µl	74.4	65.1	1.355
8.	**30 µl**	70 µl	77.5	77.0	1.358
9.	**20 µl**	80 µl	106.2	87.6	1.361
10.	**10 µl**	90 µl	109.7	105.8	1.365
11.	**0 µl**	100 µl	129.0	111.5	1.378

Table 2: Refractive index of blood samples from healthy individuals and diseased samples at four different processing stages of blood at 22°C. Data represented here is mean ± SD.

S. No.	Blood Processing stages	Samples (No. of individuals screened)	Refractive index (mean ± SD)
1	**Whole blood**	Control (41)	1.374 ± 0.0061
		HbS (10)	1.364 ± 0.0039
		HbE (15)	1.365 ± 0.0042
		HbD-Punjab (3)	1.367 ± 0.0032
		HbE-β-Thalassemia (3)	1.362 ± 0.0056
		β-Thalassemia (24)	1.364 ± 0.0044
2	**Plasma**	Control (43)	1.350 ± 0.0029
		HbS (11)	1.351 ± 0.0019
		HbE (12)	1.351 ± 0.0024
		HbD-Punjab (4)	1.350 ± 0.0015
		HbE-β-Thalassemia (4)	1.352 ± 0.0028
		β-Thalassemia (26)	1.351 ± 0.0026
3	**Hemolysate**	Control (40)	1.351 ± 0.0169
		HbS (12)	1.340 ± 0.0028
		HbE (12)	1.340 ± 0.0028
		HbD-Punjab (2)	1.342 ± 0.0021
		HbE-β-Thalassemia (2)	1.339 ± 0.0021
		β-Thalassemia (26)	1.342 ± 0.0016
4	**Pure Hb (isolated by Hemoglobind)**	Control (20)	1.337 ± 0.0022
		HbS (15)	1.335 ± 0.0009
		HbE (11)	1.335 ± 0.0003
		HbD-Punjab (2)	1.355 ± 0.0
		HbE-β-Thalassemia (3)	1.355 ± 0.0
		β-Thalassemia (6)	1.355 ± 0.0

Figure Legends

Figure 1: A standard curve showing the relationship between the refractive index and the protein concentration in whole blood at 22 °C.

Figure 2: **A comparison of refractive index of blood samples at four different processing stages at 22°C. A)** whole blood samples **B)** plasma samples **C)** hemolysate samples **D)** pure Hb samples. Data represented here as bar diagrams showing mean ± SD, *** P<0.0001, **p<0.001, *P<0.02 analyzed by one way ANOVA GraphPad Prism software. The ∗ indicate significance, while NS indicates not significant.

Figure 1.

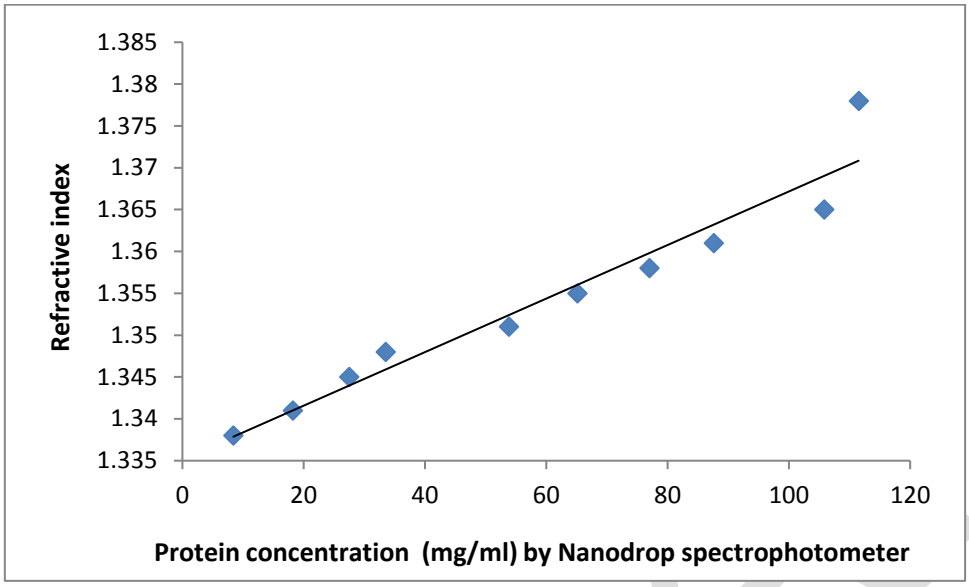

Figure 2.