ESTIMATION OF HAEMOGLOBIN USING A CYANIDE-FREE THERMAL LYSING METHOD

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Haemoglobin (Hb) is the oxygen carrying molecule from lungs to body tissues. Determination of Hb concentration is important in the detection, evaluation and management of anaemia. The gold standard method for Hb estimation is the direct Cyanmethaemoglobin (HiCN) method which uses a reagent containing Cyanide and it is known to be hazardous to the environment and the laboratory personnel. The main objective of this study was to develop a cost-effective and non-hazardous method for Hb estimation and to determine the correlation between Hb concentrations measured via a new method (Cyanide-free Thermal Lysing method) and HiCN method. The Cyanide-free Thermal Lysing method (CFTL) is based on the thermal lysis of red blood cells mixed with 0.9% (W/V) normal saline in the ratio of 1:201 and the measurement of absorbance of the haemoglobin solution by spectrophotometry. Ethylenediaminetetraacetic acid (EDTA) anticoagulated blood samples were used in the study. To determine optimal temperature, time of incubation and wavelength for CFTL method, separate test tubes were prepared by mixing 20 µl of blood with 4ml of normal saline to be incubated either at 1) 55 °C, 2) 60 °C, or 3) 65 °C for 15, 30, 45 and 60 minutes separately. Following incubation, tubes were centrifuged at 2500 rpm for 10 minutes and the absorbance spectrum of Hb solution was scanned with a blank of normal saline in visible range. After finding the optimal temperature (60 °C), optimal incubation time (30 minutes) and the optimal wavelength (539 nm) for measurement of absorbance, standard curves were prepared for HiCN method and CFTL method. Hb concentrations of 85 blood samples were estimated by both methods and statistically compared by using Pearson's correlation coefficient and paired sample t-test. There was a significant and positive correlation between the two methods ($R = 0.992$, $p < 0.001$). Paired t-test showed that there was no significant difference between the two methods ($p = 0.096$, $p > 0.05$). It takes about 45 minutes by CFTL method while 15 minutes by HiCN method for Hb estimation. Since the haemolysate is unstable, readings should be taken within five minutes following centrifugation. CFTL method could be used as an alternative technique for Hb estimation. It will reduce the cost of reagents and the toxic effects for the users and the environment.

Keywords: Haemoglobin estimation; cyanmethaemoglobin method; non-cyanide method; thermal lysis; cyanide toxicity

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INTRODUCTION

Haemoglobin (Hb) is the oxygen carrying molecule in red blood cells (RBC) from lungs to body tissues. The measurement of Hb concentration in blood plays a central role in the detection, evaluation and management of chronic and acute anaemia (Barker *et al*., 2016). The Hb measurement is also essential for screening the eligibility of donors in blood banks (Whitehead *et al*., 2019). The World Health Organization (WHO) defines the normal ranges of Hb concentration as 13.5 - 17.5 g dL^{-1} for males and 12.5 - 15.5 g dL^{-1} for females in adults (Chattopadhyay *et al*., 2021).

Anaemia is a condition in which the number of RBCs or the Hb concentration within them is lower than normal. WHO estimates that 42% of children less than 5 years of age and 40% of pregnant women worldwide are anaemic (WHO, 2023).

Direct Cyanmethaemoglobin (HiCN) method has been the gold standard for Hb estimation (Srivastava *et al*., 2014). Its advantage is the availability of a stable and internationally accepted reference standards. However, its use may create a problem, as the waste disposal of large volumes of reagent containing cyanide may constitute a potential toxic hazard (Lewis *et al*., 1991). It is known to be hazardous to the environment and occasionally harmful to the laboratory personnel also (Chakravarthy *et al*., 2012).

Earlier, there were qualitative methods such as Copper Sulphate technique for assessing Hb in clinical settings followed by quantitative methods including HiCN method (Whitehead *et al*., 2019). Other common methods are Sahli's acid haematin method, HemoCue method, Automated Haematology analysers and non-invasive pulse co-oximetry method. Analysers are although highly accurate and versatile, require good equipment, quality control, laboratory setup and trained personnel for proper functioning (Thakkar *et al*., 2021). Each method has its' advantages and limitations also. Different laboratories use different methods depending on the laboratory location, number of patients, availability of technical staff, problem of electricity and affordability (Jain, 2020). Unavailability of reagents and expensive equipment is a common problem that has to be faced by many under-resourced laboratories in developing countries.

The main objective of this study was to develop a cost-effective method for Hb estimation which is comparable to HiCN method by thermal lysis of RBCs.

Specific objectives:

- To determine a suitable wavelength for the measurement of Hb by new method using UV-Visible spectrophotometer.
- To determine suitable temperature and time of incubation of samples in order to obtain adequate concentration of Hb by haemolysis.
- To prepare a standard curve (absorbance versus concentration) for the new method.
- To determine Hb concentrations of patient samples by HiCN method and new method.

METHODOLOGY

Ethical clearance was obtained from the Ethics Review Committee, Faculty of Allied Health Sciences (FAHS), University of Ruhuna, Sri Lanka (Ref no: 242.05.2023). EDTA blood samples were collected randomly and immediately after testing from the Haematology Laboratory, Teaching Hospital-Karapitiya (THK), Galle. The experiment was performed at the Department of Medical Laboratory Science, FAHS, University of Ruhuna. Hb estimation was done by conventional HiCN method and Cyanide-free Thermal Lysing (CFTL) method simultaneously.

Cyanmethaemoglobin (HiCN) method

Hb standard solution (15.9 g dL^{-1}) was received from the Medical Research Institute, Sri Lanka. Its suitable dilutions were made in Drabkin's reagent to yield final concentrations of Hb ranging from 4 g dL^{-1} to 15.9 g dL^{-1} (Dacie and Lewis, 2017). Absorbance values of the dilution series were measured at 540nm against Drabkin's reagent blank. The graph was plotted as 'Absorbance' in Y-axis and 'Hb concentration (g/dL)' in X-axis in order to prepare the standard curve for the HiCN method. To estimate Hb concentration of unknown samples, 20 μL of blood was mixed with 5mL of Drabkin's reagent in test tubes and allowed to stand for 10 minutes at room temperature.The absorbance of the final solution was measured at 540nm against Drabkin's reagent blank. The Standard curve/graph was used to determine the Hb concentrations of unknown samples.

Cyanide-free Thermal Lysing (CFTL) method

Twelve test tubes were taken and labeled separately. In each tube, 20 µL of blood was mixed with 4.0mL of 0.9% (W/V) normal saline. Then, tubes were incubated at different temperatures for different time intervals as explained in the Table 1.

Table 1. Temperatures and incubation time periods

Temperature (°C)	Incubation periods (minutes)			
1) 55	15	30	45	60
2) 60	15	30	45	60
3) 65	15	30	45	60

During the incubation period, tubes were shaken at every 10 minutes intervals using a vortex mixer. Each tube was then centrifuged at 2500 rpm for 10 minutes (Chalmers and Russell, 1974). Immediately, the supernatant was transferred into a cuvette and absorbance spectrum was scanned in the spectrophotometer between 400nm – 700nm wavelength range. Wet films were prepared from the deposits of blood following centrifugation and observed under the microscope (Olympus CX21) with 40x objective to see the degree of haemolysis at optimal conditions.

To prepare a standard curve for CFTL method, a blood sample with a high Hb value was selected. A two-fold dilution series was prepared by mixing blood with normal saline. First, Hb values of the dilution series were determined by HiCN method. After that, 20 μL of each dilution was mixed with 4.0mL of normal saline in test tubes. After proper mixing, the tubes were incubated at 60°C for 30 mins (optimal conditions) in the water bath. All the tubes were vortexed 3 times at 10 min intervals during the incubation period. Following incubation, tubes were centrifuged at 2500 rpm for 10 mins. The absorbance of the clear supernatant was measured immediately in duplicate at 539 nm (optimal wavelength) against a blank of normal saline and average absorbance value was calculated. The standard curve was constructed by plotting the average absorbance values against their Hb concentrations.

Finally, the absorbance values of 85 EDTA samples were measured by both methods and Hb value of each sample was estimated by referring to their standard curves separately. Data was entered into SPSS (Statistical Package for the Social Sciences) software version 23 and statistically analysed by using Pearson's correlation coefficient and paired sample t-test.

RESULTS AND DISCUSSION

There was a significant increase in the absorbance at 55°C and a significant reduction at 65°C with the time of incubation. However, the highest absorbance values were seen at 60°C after 30 mins of incubation of blood-normal saline mixture (Fig. 1). At that time, the highest absorbance value was

detected at 539nm and it was used as the optimal wavelength for the CFTL method. Since the haemolysate is unstable, readings should be taken within 05 minutes following centrifugation.

The standard curves between absorbance and Hb concentration, prepared for the HiCN method and CFTL method are shown in Fig. 2 and 3, respectively. Hb values from immediate absorbance by CFTL method showed a good correlation with HiCN method ($R = 0.992$, $p < 0.01$). The results of paired sample t-test were, $t_{(84)} = -1.686$ with corresponding p value = 0.096, which is > 0.05 (two-tailed). This suggests that there is no significant difference between the two methods. The mean Hb concentrations by HiCN method and CFTL method were 11.48 ± 2 (2.16) g dL^{-1} and 11.53 ± 2 (2.18) g dL^{-1} respectively. The mean difference between both methods was only -0.049 g dL^{-1} (95% confidence interval [CI]: -0.11 to 0.01).

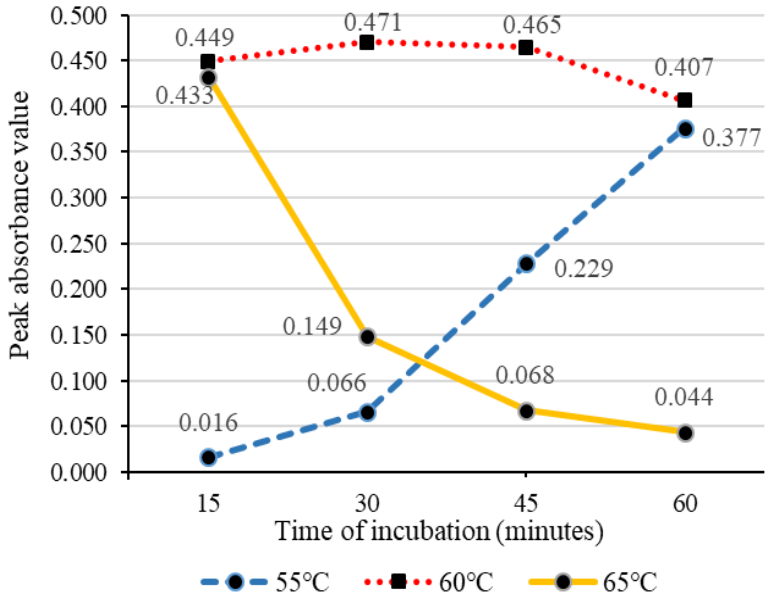

Figure 1: The average peak absorbance values obtained after incubation of samples at different temperatures (55 ℃, 60 ℃, and 65 ℃) for different time periods (15, 30, 45 and 60 minutes)

The International Committee for Standardization in Haematology (ICSH) has recommended the Drabkin as the method of choice and has suggested that all the other methods should be adjusted to be comparable to this method (Jain, 2020). It includes all forms of Hb with the exception of Sulfhaemoglobin (Weatherburn *et al*., 1962). However, abnormal plasma proteins and high leucocyte count cause turbidity on dilution of blood with Drabkin's solution giving an inaccurate estimate when absorbance is measured at 540nm (Srivastava *et al*., 2014).

Ealier, several studies have been done on haemolysis by heat. According to a study done by Eric Ponder (1949), haemolysis occurs at temperatures above 49.6℃ in the case of washed human red cells. Chalmers and Russell (1974) confirmed that haemolysis occurs at 50℃ and the amount of haemolysis increases linearly with the time of incubation. By considering that clue, the present study was conducted to lyse red cells at different temperatures above 50℃. The basis of CFTL method is the lysing of RBCs mixed with 0.9% (W/V) normal saline in the ratio of 1:201 by exposure to heat and measurement of absorbance of clear supernatant at 539nm by spectrophotometry.

Following incubation, haemolysate was centrifuged at 2500 rpm for 10 mins. As this centrifugation step was included in this procedure before measuring absorbance, it could overcome the effect of turbidity due to high plasma proteins and leucocyte counts on measurements.

There were many studies conducted on the effect of heat on RBCs in vitro. However, this is the first study which used the effect of heat to develop a Hb estimation method comparable to HiCN method.

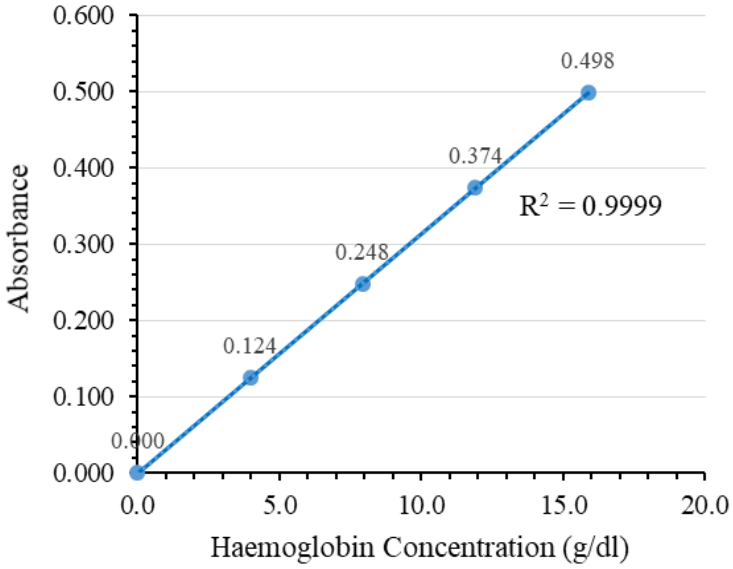

Figure 2: Standard curve of the HiCN method between Absorbance & Hb concentrations ranging from 4 g/dL to 15.9 g/dL

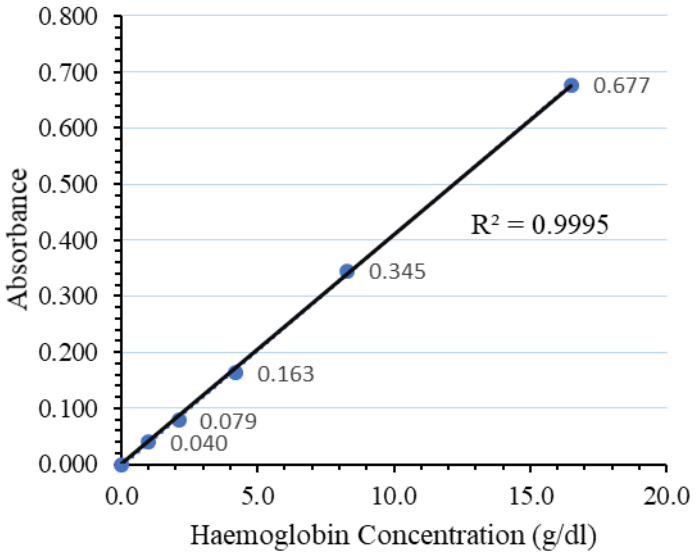

Figure 3: Standard curve of the CFTL method between Absorbance & Hb concentrations ranging from 1 g/dL to 16.5 g/dL

CONCLUSIONS

CFTL method could be used as an alternative technique for Hb estimation. It should be validated using an increased number of samples with high, low and normal Hb values. As this Hb solution is unstable, its' colour fades with time. Therefore, readings should be made immediately following centrifugation. It takes about 45 minutes by CFTL method while 15 minutes by HiCN method for Hb estimation. Reagent used is non-biohazardous and the cost is lower than Drabkin's reagent. So, this new method is safe and economical.

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