

Evaluation of the alkaline haematin D-575 method for haemoglobin estimation in east Africa*

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In many health facilities in east Africa, haemoglobin estimation is performed using visual colour comparison methods. Efforts to establish colorimetric methods face numerous constraints, including the unavailability of standards for quality control. In contrast, the alkaline haematin D-575 method for haemoglobin estimation is a colorimetric method that uses primary standards prepared from pure, crystalline chlorohaemin.

There is no significant difference in the accuracy of the alkaline haematin D-575 method and that of the reference haemiglobincyanide method ($P > 0.05$), and the response of the alkaline haematin D-575 method is linear for serially diluted blood samples over the haemoglobin concentration range 19.6–3.3 g/dl ($r = 0.994$, $y = 1.01x - 0.3$). The method has a precision of ± 0.3 g/dl (coefficient of variation = 1.8%) for whole blood, and is suitable for use with fixed-wavelength haemoglobinometers ($\lambda = 565$ nm) or with colorimeters at $\lambda = 580$ nm. Stable quality control standards could be prepared at provincial, zonal, or reference laboratories and distributed regularly to outlying laboratories.

Introduction

Haemoglobin estimation is an essential test carried out in laboratories in east Africa for the diagnosis and management of anaemia. Many current methods used for this purpose depend on visual colour comparison,^a are inaccurate, subject to user variability, and cannot be reliably used to confirm the diagnosis or follow-up treatment of anaemia. In contrast, colorimetric methods are accurate and objective.

Several colorimetric methods for haemoglobin estimation have been developed, and the haemiglobincyanide (cyanmethaemoglobin) method is currently recommended by the International Committee of Standardization in Haematology (ICSH) as the reference method since all haemoglobin derivatives (except sulphaemoglobin) are converted into a stable

end-product (haemiglobincyanide); and stable standard solutions are commercially available.

However, the haemiglobincyanide method has the following disadvantages: it employs Drabkin's reagent, which contains potassium cyanide and is photosensitive. Control standards are prepared from blood, and haemoglobin values are assigned indirectly by spectrophotometry (2).

Newer colorimetric methods of haemoglobin estimation determine lysed blood directly in a haemoglobinometer without dilution. However, haemoglobinometers are expensive and employ disposable cuvettes, making them inappropriate for widespread use in developing countries.

In the alkaline haematin D-575 (AHD-575) method of haemoglobin estimation, blood is diluted using an alkaline solution containing a non-ionic detergent. All haemoglobin derivatives are converted into a stable end-product, alkaline haematin D-575, whose absorption maximum is at $\lambda = 575$ nm (3). The AHD-575 method is standardized using chlorohaemin, a crystalline compound that is stable, well-defined, and can be obtained in pure form. When dissolved in an alkaline solution containing a non-ionic detergent, chlorohaemin forms alkaline haematin D-575, whose absorption coefficient at $\lambda = 575$ nm is $6.9601 \text{ mmol}^{-1} \cdot \text{cm}^{-1}$. Alkaline haematin methods in which blood is diluted using alkaline solutions without detergent have been described; however, in

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these methods, certain forms of haemoglobin are resistant to alkali denaturation, notably fetal haemoglobin (HbF) (5).

In this article, haemoglobin estimation using the AHD-575 method is evaluated for possible introduction into laboratories in east Africa.

Materials and methods

The alkaline haematin-D (AHD) reagent was prepared by dissolving 25.0 g of Triton X-100^b in 1 l of 0.1 mol/l sodium hydroxide (GPR grade). The control standard (AHD standard) was prepared by dissolving 36 mg of chlorohaemin ($M_r = 651.95$) in 10.0 ml of the AHD reagent (5.585 mmol/l). At a 1:151 dilution this control standard has an absorbance of 0.26 at $\lambda = 575$ nm, equivalent to a blood sample of haemoglobin concentration 9.0 g/dl.

Drabkin's reagent was obtained from the haemoglobin kit manufactured by Compur (RCM test)^c 10-ml vials of the haemiglobincyanide standard (572 mg/l, batch number 36210-9371550K; expiry date July 1994) were obtained from BDH.^d

The haemiglobincyanide standard solution (572 mg/l) is equivalent to a blood sample containing

$$\frac{572 \times 251}{10 \times 1000} = 14.4 \text{ g/dl of haemoglobin,}$$

where

1000 is the conversion factor for mg to g;
10 is the conversion factor for g/l to g/dl; and
251 is the dilution factor.

The accuracy of the AHD-575 method was evaluated as outlined below.

A total of 100 whole blood samples, anticoagulated in ethylenediaminetetraacetic acid (EDTA), were obtained from a hospital in Nairobi, over a 1-week period. These samples were diluted in duplicate in AHD reagent (1:151) and in Drabkin's reagent (1:251) by carefully pipetting (using calibrated capillary tubes) 10 μ l of blood into 1.5 ml of the AHD reagent and 5 μ l of blood into 1.25 ml of Drabkin's reagent, respectively. The alkaline haematin control standard was similarly diluted 1:151, by pipetting 10 μ l of the standard into 1.5 ml of the AHD reagent.

The absorbances of the standard and blood samples diluted in the AHD reagent were read against the AHD reagent blank in a WPA CO 700D color-

imeter, using a filter with peak transmission at $\lambda = 580$ nm (No. 606). Similarly, the absorbances of the haemiglobincyanide standard and blood samples diluted in Drabkin's reagent were read against the reagent blank in the same colorimeter using a filter with peak transmission at $\lambda = 550$ nm (No. 605, yellow/green).

The absorbances obtained were converted into haemoglobin concentrations (g/dl) using the following relationship:

Haemoglobin concentration of test sample =

$$\frac{A_t}{A_s} \times \text{concentration of standard}$$

where A_t = absorbance of the test sample and
 A_s = absorbance of the standard.

The linearity of the AHD-575 method was evaluated as follows:

- About 50 ml of whole blood from an expired unit of blood was obtained and centrifuged for 10 minutes in an electric centrifuge (approx. 5000 rpm).
- Two-thirds of the plasma was then removed and stored and the blood sample was resuspended by being mixed for 2 minutes using a laboratory shaker (Vibrofix VF-1) at 1000 rpm.
- The haemoglobin concentration of the remaining blood sample was estimated in duplicate using the AHD-575 method and the average concentration of the sample calculated.
- Duplicate dilutions of the blood in plasma (blood: plasma = 5:6, 4:6, 3:6, 2:6, and 1:6), corresponding to a nominal haemoglobin concentration range of 19.6–3.3 g/dl, were prepared and the haemoglobin concentrations were estimated using the AHD-575 method in duplicate; the average concentration for each dilution was calculated.

The precision of the AHD-575 method was evaluated as follows:

- The haemoglobin concentration of a 5-ml sample of fresh venous blood sample anticoagulated in EDTA was determined using the AHD-575 method.
- The sample was then stored in a refrigerator at 4–8 °C and repeat determinations (ten times) were made once a day over a period of 2 weeks.

The stability of the alkaline haematin D-575 reagent at room temperature was assessed by preparing solutions of alkaline haematin and haemiglobincyanide from three samples of whole blood, and comparing the absorbances at different time intervals over a 24-hour period.

The ability of the AHD reagent to denature HbF was assessed by comparing the haemoglobin of three

^b Aldrich Chemical Co. Ltd., Gillingham, Dorset, England.

^c Compur—Electronic GmbH, Munich, Germany.

^d BDH, Laboratory Supplies, Poole, Dorset, England.

samples of cord blood determined using the AHD-575 and the haemiglobincyanide methods.

The stability of the AHD-575 control standard was assessed by comparing the absorbances of the standard and the AHD reagent blank throughout the study. Subsequently the control standard was stored at 4–8 °C and the absorbances of the standard versus the blank were determined 14 times over an 8-month period from the date of preparation.

The use of the AHD-575 method with a Cynox haemoglobinometer designed for haemoglobin estimation using the haemiglobincyanide method was assessed by comparing the haemoglobin levels of 10 samples of whole blood using the haemoglobinometer and the WPA CO 700D colorimeter (at $\lambda = 580 \text{ nm}$).

Results

There was good correlation between the haemoglobin levels of the 100 blood samples analysed using the AHD-575 and haemiglobincyanide methods (Fig. 1).

For the blood samples that were diluted serially from 19.6 g/dl to 3.3 g/dl whose haemoglobin concentrations were determined using the AHD-575 method, Fig. 2 shows the regression plot of the expected versus measured haemoglobin concentrations.

The results of the repeat analysis ($\times 10$) of the 5-ml blood sample determined over a period of 2 weeks using the AHD-575 method were as follows:

— Mean haemoglobin level = $16.9 \pm 0.3 \text{ g/dl}$; coefficient of variation (CV) = 1.8%.

Table 1 shows the haemoglobin concentrations of alkaline haematin prepared from three samples

Fig. 1. Correlation plot for haemoglobin levels (g/dl) in 100 blood samples, determined using the AHD-575 and the haemiglobincyanide (HbCN) methods.

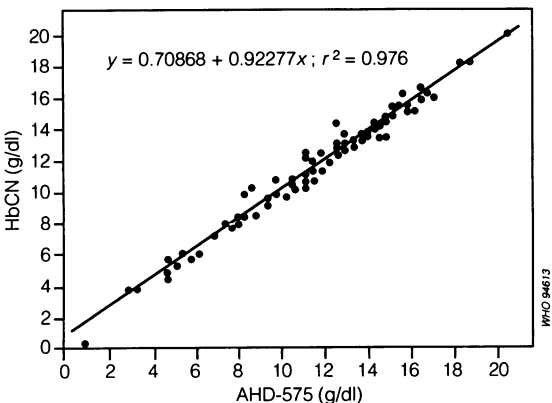
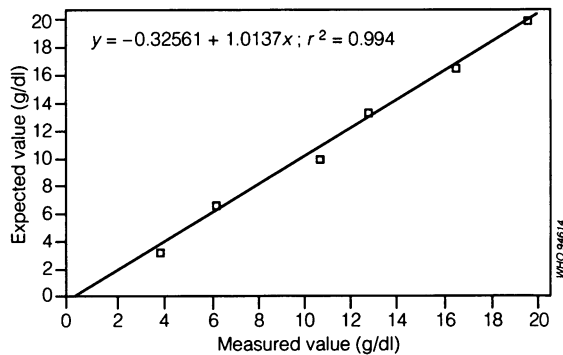


Table 1: Concentrations of haemoglobin determined in three samples of whole blood using the AHD-575 and haemiglobincyanide methods

Time	Sample:		
	1	2	3
	Haemoglobin concentration (g/dl)		
11:30	14.7 (14.8) ^a	15.2 (14.9)	15.2 (14.9)
12:00	14.7 (14.8)	14.7 (14.9)	15.2 (14.9)
12:30	14.7 (14.9)	15.2 (14.9)	15.2 (14.8)
13:00	14.2 (14.8)	14.7 (14.9)	15.6 (14.8)
14:00	15.2 (14.8)	14.7 (14.9)	15.2 (14.9)
15:00	14.7 (14.9)	15.2 (14.9)	15.2 (14.8)
17:00	14.7 (15.0)	15.2 (14.9)	15.6 (14.8)
08:00 (next day)	14.7 (15.0)	15.2 (14.9)	15.6 (14.9)

^a Figures in parentheses are the levels determined using the haemiglobincyanide method.

Fig. 2. Correlation plot for the expected versus measured haemoglobin levels (g/dl) obtained using the AHD-575 method.



of whole blood, determined repeatedly at different time intervals using the AHD-575 method and the haemiglobincyanide method.

Table 2 shows the haemoglobin concentrations of the three cord blood samples that were determined using the AHD-575 and the haemiglobincyanide methods.

Table 2: Haemoglobin concentrations in cord blood samples determined using the AHD-575 and haemiglobincyanide methods

Sample	Haemoglobin concentration (g/dl):	
	AHD-575	Haemiglobincyanide
1	14.3	14.2
2	14.3	14.4
3	13.8	13.8

The mean absorbance of the AHD standard (0.36 g/l) that was stored at 4–8 °C (1:151 dilution) and measured 14 times using a WPA CO 700D colorimeter versus the AHD reagent blank over a period of 8 months was 0.26 ± 0.01 ; CV = 3.8%.

The correlation between the haemoglobin concentrations (g/dl) of 10 blood samples determined using the AHD-575 method with a Cynox haemoglobinometer and with a WPA CO 700D colorimeter ($\lambda = 580$ nm) is shown in Fig. 3.

For 5000 haemoglobin investigations the total costs of the reagents and standards required for the AHD-575 method and haemiglobincyanide method (1:251) were US\$ 7.40 and US\$ 28.70, respectively, while the costs of the respective reagents only were US\$ 6.90 and US\$ 0.80.

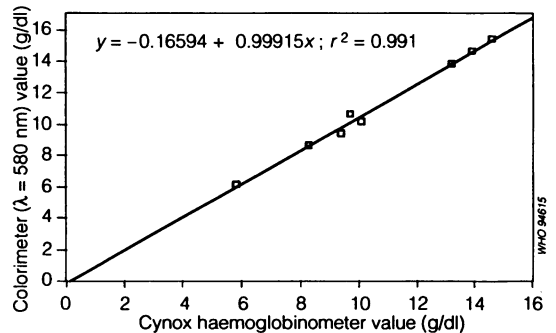
Discussion

Haemoglobin estimation is one of the most frequently used procedures in clinical laboratories at all levels in east Africa. It is essential for diagnosing anaemia, following up the management of anaemic patients, and screening for anaemia among blood donors and patients attending antenatal clinics. Haemoglobin estimation is also included in routine medical examinations of individuals before taking up employment or enrolling in institutions.

For haemoglobin estimation, many laboratories in east Africa still use visual colour comparisons, e.g., the Lovibond, Sahli, and Tallqvist methods, or the BMS visual haemoglobinometer. Such methods are subjective and are not sufficiently accurate for the diagnosis or management of anaemia.⁶

A few laboratories in east Africa use colorimetric methods to estimate haemoglobin; for example, the oxyhaemoglobin and haemiglobincyanide methods, in which a small volume of blood is diluted using a weak solution of ammonium hydroxide or Drabkin's reagent and the resulting coloured solution is determined using a haemoglobinometer or colorimeter. The oxyhaemoglobin method has the advantage that it uses a cheap reagent that is easy to prepare; however, all forms of haemoglobin are not converted into oxyhaemoglobin and the pigment formed decomposes within 1 hour of preparation, requiring the results to be read without delay. More importantly, there are no stable standards for quality control purposes (1). The haemiglobincyanide method is the recommended international reference procedure for haemoglobin estimation. Most forms of haemoglobin are converted into haemiglobincyanide, and since the pigment formed is stable, quality

Fig. 3. Correlation plot for the haemoglobin levels (g/dl) determined using a colorimeter and a Cynox haemoglobinometer.



control standards are available. However, Drabkin's reagent contains potassium cyanide, which is very toxic and increasingly difficult to obtain, and the solution is photosensitive. The quality control standards are solutions of haemiglobincyanide, which have been indirectly determined by spectrophotometry in selected reference laboratories (2). These standards are also expensive and not readily available in east Africa.

A novel lauryl sulfate haemoglobin colorimetric method has been described and evaluated for use in automated analysers (6). The pigment formed is stable for only 4 hours at room temperature, and hence the method is not ideal for manual use in busy health facilities. Also, no standards are available.

Oxyhaemoglobin and haemiglobincyanide pigments have absorption maxima at approximately the same wavelength. Thus, the haemoglobinometers in current use can be used for either method. Such haemoglobinometers are sometimes supplied with coloured filters for quality control purposes; however, the filters are usually detachable and are often lost or become scratched.

Newer haemoglobinometers, which measure the haemoglobin concentration of lysed blood directly without dilution, are now available; however, they are expensive and use disposable cuvettes, both of which make them unsuitable for use in developing countries.

The AHD-575 method, like the oxyhaemoglobin and haemiglobincyanide methods, involves diluting small volumes of blood (which must be accurately pipetted) in a lysing solution (AHD reagent) prior to colorimetric measurement. The AHD-575 reagent is not toxic or photosensitive, and all forms of haemoglobin are converted into alkaline haematin D; also, we found that the AHD reagent was stable for 8 months at 4–8 °C, which agrees with the findings of

⁶ See footnote a, p. 937.

Zander et al.; the concentration of Triton X-100 can be varied over the range 25–50 g/l and that of sodium hydroxide over the range 0.01–0.1 mmol/l without altering the test results (3, 4). Thus the AHD reagent can be prepared without the necessity of making precise measurements. Chlorohaemin is a stable compound that can be obtained in pure form and primary standards for the AHD-575 method can be prepared from it. The standards are stable for 8 months at 4–8 °C. The cost of the basic reagents for the AHD-575 method are greater than that of those for the haemiglobincyanide method, largely due to the high cost of Triton X-100. Less costly, suitable detergents should therefore be sought for the AHD-575 method. However, the cost of the AHD-575 standard is considerably less than that of commercial haemiglobincyanide standards, making the total cost of the AHD-575 method significantly lower. The AHD-575 standard could be prepared in provincial (regional) laboratories, zonal hospital laboratories, or national reference laboratories in east Africa and distributed to peripheral laboratories. The AHD-575 method therefore offers the possibility of safe and quality haemoglobinometry at all levels of health facility at a reduced cost.

The maximum absorption of the alkaline haematin D pigment differs from that of oxyhaemoglobin or haemiglobincyanide. However, some haemoglobinometers designed for the haemiglobincyanide method are suitable for use also with the alkaline haematin D-575 method, e.g., the Cynox haemoglobinometer is fitted with a light-emitting diode (LED) that emits light at $\lambda = 565$ nm. Many newer haemoglobinometers (e.g., Compur, ZJY-1, etc) are also equipped with LEDs and health facilities that already have such instruments can therefore easily use the AHD-575 method.

The advantages of the AHD-575 method clearly warrant its introduction into health facilities in east Africa and in developing countries in other areas.

Acknowledgements

We thank WHO for encouraging us to perform the evaluation and Prof. Wolf for providing the chlorohaemin crystals.

Résumé

Evaluation de la méthode à l'hématine alcaline D-575 pour le dosage de l'hémoglobine en Afrique de l'Est

Dans beaucoup de centres de santé d'Afrique de l'Est, le dosage de l'hémoglobine se fait par des méthodes de comparaison visuelle. La mise au

point de méthodes colorimétriques se heurte à de nombreuses contraintes, dont l'absence d'étalons pour le contrôle de qualité. La méthode à l'hématine alcaline D-575 est une méthode colorimétrique qui fait appel à des étalons primaires préparés à partir de chlorohémine pure cristallisée.

Il n'y a pas de différence significative pour ce qui est de l'exactitude entre cette méthode et la méthode de référence à la cyanométhémoglobine ($P > 0,05$). L'analyse d'une série de dilutions de prélèvements sanguins a montré que la méthode à l'hématine alcaline D-575 donnait une réponse linéaire dans la plage de concentrations comprise entre 19,6 et 3,3 g/l d'hémoglobine ($r = 0,994$, $y = 1,01x - 0,3$) et sa précision a été évaluée à 0,3 g/l (coefficient de variation CV = 1,8%) pour le sang total.

L'étalon de référence est stable pendant huit mois (ET = 0,01, CV = 3,8%) à 4–8 °C et l'hématine alcaline est stable pendant 24 heures à 22 °C.

La méthode à l'hématine alcaline D-575 peut être utilisée avec des hémoglobinomètres à longueur d'onde fixe ($\lambda = 565$ nm) prévus pour la méthode à la cyanométhémoglobine, ou avec des colorimètres fonctionnant à 580 nm. Des étalons stables pour le contrôle de la qualité peuvent être préparés dans des laboratoires provinciaux, régionaux ou de référence et distribués régulièrement aux laboratoires périphériques. La méthode est bien adaptée aux besoins des pays d'Afrique de l'Est et des pays en développement d'autres régions.

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