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Original Article

Pathology Section

Haemoglobin Estimation by Non-cyanide Methods

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ABSTRACT

Introduction Haemoglobin is an oxygen carrying protein in the red blood cells. It is estimated by the haemoglobincyanide method (HiCN). This method is known to be hazardous to the environment and occasionally harmful to the laboratory personnel also.

Aims and Objectives: To compare and study the haemoglobin concentrations which were estimated by cyanide and non-cyanide methods. To suggest the advantages of the cyanide free methods over the cyanide methods. To assess whether these methods were better as compared to the haemoglobincyanide method, for medical teaching, blood bank camps and for laboratory investigations.

Materials and Methods: 2000 samples in which haemoglobin estimation was done by the haemoglobincyanide method, the Sodium Lauryl Sulphate method and a modified alkaline haematin method which used cetrimide were compared and statistically analyzed by using Pearson's correlation coefficient.

Results: The haemoglobin concentrations which were estimated by the Sodium Lauryl Sulphate method and the modified alkaline haematin method were similar to those which were obtained by the conventional cyanmethaemoglobin method, with r=0.986 and r=0.996 respectively.

Conclusion: The modified alkaline haematin method was as accurate as the cyanmethaemoglobin method. It was economical. The toxic effects of cyanide can be prevented by using this method commercially.

Key Words: Haemoglobincyanide, Cetrimide, Sodium Lauryl Sulphate

INTRODUCTION

Haemoglobin is an iron containing, oxygen-transport metalloprotein in the red blood cells of all the vertebrates (with the exception of the fish family, Channichthyidae) [1] as well as in the tissues of some invertebrates [1]. The haemoglobin in the blood carries oxygen from the respiratory organs to the rest of the body [2].

Haemoglobin is estimated by the haemoglobincyanide method (HiCN) in laboratories, as it is a relatively stable method and as it has an internationally accepted reference standard calibrator [2, 3], but the potassium cyanide which is used in the haemoglobincyanide method is toxic at high concentrations, making the management of the safe disposal of this reagent difficult [4]. Automated cell counters now use Sodium lauryl sulphate (SLS) instead of toxic reagents [5]. The commercially available Hemosafe solution (which contains sodium lauryl sulphate) is less stable as compared to the Drabkin solution, the reagent which is used in HiCN [5]. Hence, for the haemoglobin assay, non toxic, economical and more stable chemicals are needed [4].

2 Non-cyanide methods, the Sodium lauryl sulphate (SLS) method and the modified alkaline haematin method (MAH) which uses sodium tetraborate and ceramide have been chosen to evaluate haemoglobin with respect to the cost, standard results, stability and safety in comparison to the haemoglobincyanide method. If the haemoglobin values which are obtained by these methods are comparable to those which are derived from the standard haemoglobincyanide method, these cyanide free methods can be insisted to be used in teaching institutions, blood bank camps and laboratories, to avoid biohazards to the their staff and the environment.

MATERIALS AND METHODS

This study was a comparative and a hospital based study. It was conducted within 2 months in the hospital, with a sample size of 2000 randomly selected patients. Ethical clearance was obtained from hospital ethical committee. Haemoglobin was estimated by the haemoglobincyanide method, the Sodium Lauryl Sulphate method and a modified alkaline haematin method which used cetrimide. The photocolourimeter which was used for these procedures was ELICO CL 157. The photocolourimeter was standardized by repeating the same sample 10 times at different timings. The absorbance was read and the co-efficient of variation was calculated. Pipette calibration was done by pipetting the same sample 10 times and the mean , SD and the co-efficient of variation was measured.

1) The Haemoglobincyanide Method: Drabkin's reagent was obtained from Ensure Biotech Pvt.Ltd. [with a 10ml vial of the standard. (Lot no: E 19; Expiry Date: October-2012)]. The absorbance of cyanmethaemoglobin was directly proportional to the haemoglobin concentration in the sample.

Procedure: The reagent was Drabkins solution, which was ready to use. The standard absorbance was read before the start of the procedure. 0.02ml of the test sample was added to 4.0ml of Drabkin's solution [4]. The diluted sample was allowed to stand for 10 minutes, it was then transferred to a cuvette and the optical density was determined at 540nm against a blank of Drabkin's solution. The value of the sample was determined by interpolation from the graph which was obtained [4,6].

2) The Sodium Lauryl Sulphate (SLS) Method: HEMOSAFE from the Tulip group Coral containing SLS - Reagent - 1000ml.

Hemosafe SLS – Standard (60mg/dl) – 10ml: The total conversion to the SLS-Haemoglobin was rapid and SLS converted haemoglobin fully to methaemoglobin.

Procedure: 0.02ml of the test sample was added to 5.0ml of sodium lauryl sulphate. The diluted sample was allowed to stand for 5 minutes, it was then transferred to a cuvette and the optical density was determined at 540nm against a blank of the SLS solution. The value of the sample was determined by interpolation from the graph which was obtained from the standard [4,5].

3. The Modified Alkaline Hematin Method (MAH)

MATERIALS

Borate working solution Sodium tetra borate (borax) – 1.9gm/lit, Sodium hydroxide – 2gm/l, Cetyltrimethylammonium bromide – 10gm/lit. RN (Br) (CH3) R (Cetrimide) was used as lysing agent. It is a quaternary ammonium compound which is a crystalline powder with a disinfectant activity. As a modification, the original compound which we used was 0.5ml/100ml sol. of readily available SAVLON. (Cetrimide 3% w/v and Chlorhexidine Gluconate solution 0.5% v/v).

Principle: Cetrimide acts by lysing the erythrocytes and precipitating haemoglobin which conjugates with boronic acid to form a red precipitate which is read at 540nm.

Procedure: 0.02ml of the test sample was added to 3.0ml of the borate working solution. The diluted sample was allowed to stand for 5 minutes, it was then transferred to a cuvette and the optical density was determined at 540nm against a blank of the borate working solution.

A standard curve was plotted for the different haemoglobin values which were obtained by using Drabkin's solution and its standard, against the optical density which was obtained from the borate working solution. Depending on the optical density values, the results were compared with the standard curve. The stability of the modified alkaline haematin reagent at room temperature was assessed by preparing solutions of alkaline haematin and cyanmethaemoglobin from the samples of whole blood, and comparing the absorbance at different time intervals over a 24 hours period. The time which was taken for complete haemolysis was calculated by noting the absorbance values every minute and by centrifuging the samples to look for the cell buttons. There was no cell button formation from the third minute onwards. The ability to denature foetal haemoglobin (HbF) was assessed by comparing the haemoglobin values of 20 samples of cord blood by using the modified alkaline haematin reagent and Drabkin's solution.

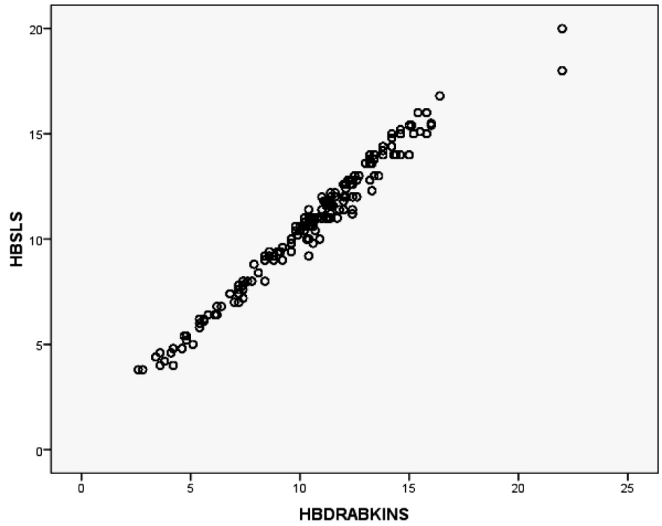
These samples were used for estimating the stability of the modified alkaline haematin reagent and the HbF and have not been included in the current data [4,7,8].

RESULTS

The haemoglobin which was estimated, ranged from 3.8gms to 20gms and the patients who were included in this study were newborns to 88year old. The optical density was measured at 540nm for all the 3 methods. The reaction completion time for the haemoglobincyanide method was 10 minutes, whereas for the sodium lauryl sulphate method and the alkaline haematin method, it was less than 3 minutes. The stability of the haemochromogens

Correlations			
		HBDRABKINS	HBSLS
HBDRABKINS	Pearson Correlation	1	.986**
	Sig. (2-tailed)		.000
	N	2000	2000
HBSLS	Pearson Correlation	.986**	1
	Sig. (2-tailed)	.000	
	N	2000	2000

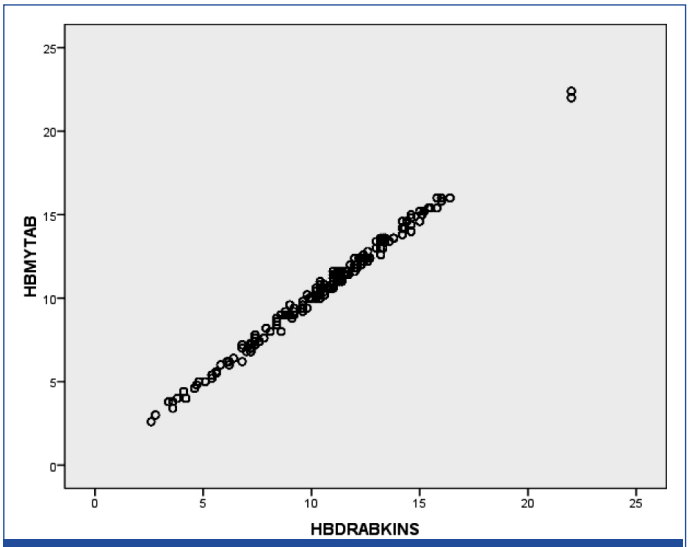
[Table/Fig-1] Correlation between Cyanmeth hemoglobin method and sodium lauryl sulphate method.



[Table/Fig-2]: Correlation between Cyanmeth hemoglobin method and sodium lauryl sulphate method.

Correlations			
		HBDRABKINS	HBMYTAB
HBDRABKINS	Pearson Correlation	1	.996**
	Sig. (2-tailed)		.000
	N	2000	2000
HBMYTAB	Pearson Correlation	.996**	1
	Sig. (2-tailed)	.000	
	N	2000	2000

[Table/Fig-3] Correlation between Cyanmeth hemoglobin method and modified alkaline hematin method.

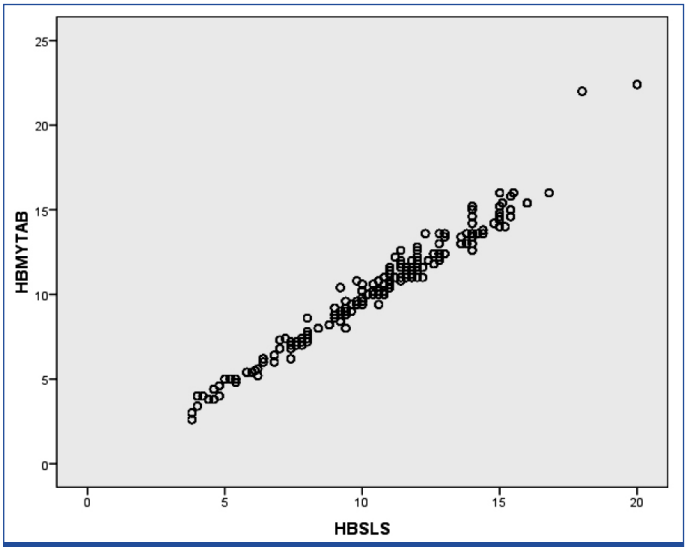


[Table/Fig-4]: Correlation between Cyanmeth hemoglobin method and modified alkaline hematin method.

which were formed by the modified alkaline haematin method, which were observed at 540nm, produced stable absorbance readings which remained unchanged till four days consecutively. The correlation between the haemoglobincyanide method, the sodium lauryl sulphate method and the alkaline haematin method was done by using the Pearson's correlation coefficient. A good correlation was observed between the non-cyanide methods and the haemoglobincyanide method. The correlation coefficients of 0.98 and 0.99 were statistically significant at p<0.000, which indicated that the calculated value, r was unlikely to have resulted from a random chance.

Correlations			
		HBMYTAB	HBSLS
HBMYTAB	Pearson Correlation	1	.982**
	Sig. (2-tailed)		.000
	N	2000	2000
HBSLS	Pearson Correlation	.982**	1
	Sig. (2-tailed)	.000	
	N	2000	2000

[Table/Fig-5] Correlation between sodium lauryl sulphate method and modified alkaline hematin method.



[Table/Fig-6]: 3 Correlation between sodium lauryl sulphate method and modified alkaline hematin method.

DISCUSSION

Haemoglobin estimation is one of the most common tests which are done at the bedsides of patients, at primary health care centres, in antenatal clinics; during the follow up of management of anaemic patients and during the screening for anaemia among blood donors [9, 10]. A good number of methods is available for haemoglobin estimation, with the colourimetric method being the most popular one. The manual HiCN method was phased out as a routine method and it gradually became a reference method [2]. Other factors like legislation which affects the transportation of the reagents and the control of substances such as cyanides which are hazardous to health, have led to the development of methods which do not employ cyanide for the determination of haemoglobin [4]. In our country, approximately 70% of the laboratories still use the manual HiCN method for Hb estimation in the rural areas [4]. The cyanmethemoglobin method has been accepted as a standard method [2] for Hb estimation. This method uses a stable standard solution which helps in deriving uniform and consistent results. But this solution contains potassium cyanide which in high quantities, makes its safe disposal difficult.

Sodium lauryl sulfate converts haemoglobin into methaemoglobin in the order of oxyhaemoglobin, haemochrome and methaemoglobin by its oxidative activity [5].Therefore, unlike other methods, this method does not need oxidative reagents and it does not generate toxic wastes such as KCN and NaN3 which cause environmental pollution [10]. The solution is photosensitive and less stable and the absorbance reading has to be taken within one hour. This solution is excellent for use in automated cell counters [5]. The alkaline haematin method (AHD-575) has been evaluated extensively by some authors in other countries and east Africa [7,8,11]. There are no Indian studies which have been done, which have compared the alkaline haematin method with HiCN [4], except for the recent study which has been done by Vinay B. Shah et al.,[4]. The alkaline haematin method had been promising, but the lysing reagent which was used was Triton X100 which had made the reagent costly. Vinay B. Shah et al., [4] used Myristyl trimethyl ammonium chloride. In our study, we tried it with commercially available Savlon (Cetrimide 3% w/v and Chlorhexidine Gluconate solution 1.5% v/v). As it is easily available and economical, this can be used in peripheral centers in the alkaline boronate method.

Both the above non-cyanide methods were compared for repeatability, reagent stability, and accuracy in correlation with the cyanmethhaemoglobin method and both the reagents gave excellent results. The quantity of the reagent which was used by the MAH was low (3ml) as compared to that which was used by the other two methods (5ml).

Both sodium lauryl sulfate and cetrimide have been used as detergents in shampoo and soap industries extensively.Sodium tetra borate is a well-known food additive and it has been thought to cause gastric symptoms while it was used in high quantities. No other definite complications have been reported.

In both the non cyanide methods, the absorbance was read at 540nm on a colourimeter. These methods can also use LED haemoglobinometers with the same accuracy.

The stability of the modified alkaline haematin reagent promises a standard preparation for this method exclusively. The present study was done on 2000 samples and the alkaline borate method was modified by using Savlon as a lysing agent, which is easily available at primary health centres. The correlation between HiCN and the MAH was excellent and it was comparable with the findings of a study which was done by Vinya B. Shah et al., [4] in 2011.

Method	Cost/test	Results C/NC	BIOTOXIC
Cyanmethemoglobin method	1 Rs.	STANDARD	YES
Sodium lauryl sulphate method	3 Rs.	CORRELATED	NO
Boronate affinity method	0.1 Rs.	CORRELATED	NO

[Table/Fig-7]: Summary of various methods of hemoglobin estimation.

CONCLUSION

The cyanmethemoglobin (HiCN) method for measuring haemoglobin is used extensively worldwide; its advantage is the ready availability of a stable and internationally accepted reference

standard/calibrator. However, its use may create a problem, as the disposal of large volumes of reagent which contains cyanide may constitute a potential bio-toxic hazard. As an alternative, the non-toxic sodium lauryl sulphate and the modified alkaline haematin methods have been used. The results are reproducible as for HiCN for measuring haemoglobin at all concentrations. Hence, these methods can be employed in primary health care centres, teaching hospitals and diagnostic laboratories.

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