AN EVALUATION OF HAEMOGLOBIN DETERMINATION USING SODIUM LAURYL SULFATE

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Abstract

Introduction: Various methods for the quantitative determination of Hb have been developed during the last decade, based on chemical and physical principles of Hb, gasometry or spectrophotometry.

Methods: Spectrophotometric measurement of Hb as HiCN was carried out by the ICSH recommended reference method and by cyanide free (sodium lauryl sulfate) reagent. Measurements were carried out in 347 venous blood collected into K_2EDTA anticoagulant. Normal samples were obtained from volunteers and other samples were obtained from patient specimens in routine diagnostic service. The latter included Leukaemia with high WBC count, Lipaemia, Cord blood, other abnormalities (uraemia, jaundice thalassemia minor and major) and methaemoglobinemia. The Hb value then determined by using HiCN method and SLS method.

Results: Maximum absorbance is at 535 nm, the conversion time of Hb to SLS-Hb was rapid of less than 15 seconds. The freshly prepared SLS-Hb was stabile in the first 120 minutes after dilution. Haemoglobin converts almost instantaneously and there is a direct relationship of absorbance to Hb concentration over awid range of measurements (r = 0.999).

Conclusion: its reliability is equal to that of HiCN method with routine blood specimens, but slightly more reliable than HiCN method when there is interference by lipaemia. There is no significant difference in measurements on samples containing HbF (r = 0.995). It measures methamoglobin with correlation coefficient (r) of 0.999. It has a major advantage in that the reagent is non-hazardous compound.

Keywords: Haemoglobin, Sodium lauryl sulphate

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Introduction

Haemoglobin is a chromoproteid[1]. It is calculated to have a molecular weight of 64 458[2]. Each red cell contains approximately 640-million haemoglobin molecule[3]. Haemoglobin possess certain chemical and physical properties, which may be used in estimating its concentration in blood[4].

The depth of red color of blood is directly proportional to the concentration of iron and the concentration of Hb present. The red color of blood, therefore may be compared with that of various preparation used as standards and the concentration of Hb determined[4].

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Various methods for the quantitative determination of Hb have been developed during the last decade, based on chemical and physical principles of Hb, gasometry or spectrophotometer.

The cyanmcthaemoglobin (HiCN) has been accepted worldwide during 1953-1963[5]. In addition, it's recommended by the International Committee of Standardization in Haematology (ICSH) in 1965 and 1967, due to the accuracy and stability of result[6-9]. However, the presence of potassium cyanide (KCN) and potassium ferricyanide (K_3-Fe $(CN)_6$) in the reagents has raised problems of laboratory and environmental pollution, and may constitute a potential toxic hazard[8].

Oshiro et al. (1982) developed cyanide free method of Hb determination that it based on a low toxicity compound sodium lauryl sulfate (SLS), a surfactant[10]. Haemoglobin is rapidly converted into SLS-Hb (10 seconds). The majority of Hb

derivatives encountered in clinical practice are converted to SLS-hemichrome complex using this method. Therefore, unlike others, this method does not need oxidative reagents and does not generate toxic wastes such as KCN and NaN3 which cause environmental pollution[11]. Therefore, the aim of this study was to evaluate sodium lauryl sulfate method for determination of haemoglobin in routine blood specimens.

Materials and Methods

This study was conducted at AL-Kadhimyia General Hospital, during the period from 4th of June 2002 till December 2002.

Spectrophotometric measurement of Hb as HiCN was carried out by the ICSH recommended reference method and by cyanide-free (sodium lauryl sulfate) reagent.

Measurements were carried out on 347 venous blood collected into K2EDTA anticoagulant (1.5mg/ml blood). Normal samples were obtained from volunteers and other samples were obtained from patients specimens in the routine diagnostic service. The latter included:

1- Leukaemias with high leukocyte counts (12 samples).
2- Lipaemias (15 samples).
3- Cord blood (35 samples).
4- Methaemoglobin (35 samples), was produced by exposing normal blood to sodium nitrate (2ml of blood to 0.1ml of sodium nitrate)[12].

The Hb value then determined by using two sets of reagents:

1. Drabkins (cyanide-ferricyanid solution) by using haemoglobincyanid (HiCN) method
2. New cyanide-free (sodium lauryl sulfate SLS) reagent (2g of SLS, 2.5g of disodium EDTA and one liter deionized distilled water).

Two types of Drabkins solution were used one supplied by Randox and other by Iraqi manufacturer; Hb value was determined by these reagents and means value then calculated.

1. Reagents and instruments:

1- Drabkins (cyanide-ferricyanid) solution one supplied by Randox and the other by Iraqi manufacturer.
2- HiCN standard so recommended by ICSH.
3- HiCN standard (Iraqi manufacturer).
4- SLS reagent (2 g of SLS, 2.5g of EDTA in one liter deionized distilled water).
5- Spectrophotometer.
6- Automated pipette.
7- Glass tube.

2. Methods:

A) HICN method[5]

Twenty microliter of EDTA blood were added to 5 ml of diluent (Drabkins solution), stopper the tube containing the solution & invert it several times, allow to stand at room temperature for about 5–15 minutes to ensure the completion of the reaction , the solution of HiCN is compared with reagent blank in spectrophotometer at 540 nm wave length in which the absorbance is read and the results obtained from stander curve , in which the stander curve prepared by measurement of HiCN reference solution (standard) by the same spectrophotometer as is to be used for the subsequent hemoglobinometry. The absorbance of the solution (standard) against blank of cyanide-ferricyanide reagent. We make reading with the same standard solution diluted with the reagent (Drabkins) 1 in 2 ,1 in 3 ,1 in 4,…etc. translate the Hb value of the solution into terms of g/l. Plot the reading on linear graph paper using arithmetical scales, with absorbance as ordinates (vertical scale). The point should fit a straight line that passes through the origin; this provides a check that the calibration of spectrophotometer is linear (assuming that the standard has been correctly diluted). We make two standard curve one for randox reagent & other for Iraqi manufactured reagent in which each one has it standard[14].

We taken the mean of duplicated measurement of each sample and tabulated

in order to be compared with the new method (SLS).

B) SLS METHOD[10]

Twenty microliter of fresh blood with EDTA was added into 3 ml of SLS reagent & read the absorbance within 20 second on spectrophotometer at 535nm wavelength.

Thus for preparing a calibration graph or standard graph it is necessary to adopt a two–stage procedure using fresh blood as an intermediate reference preparation, the Hb value of which must first be established by the HiCN method. For this purpose blood collected into APD or ACD anticoagulant is suitable for 2-3 weeks if stored at 4 c providing it remain sterile. A standard sample will maintain its assigned value for several months, especially if stored frozen.

After established of Hb value by HiCN method we make dilution by SLS reagent 1 in 2, 1 in 3, 1 in 4…. etc. Plot the reading into linear graph paper using arithmetical scale, with absorbance as ordinates (vertical scale). The points should fit astriaght line that pass through the origin. From the standard curve, we calculate the Hb value and plot in table.

Results

1. Spectrophotometry:

a. Conversion Time:

Total conversion to Hb-SLS was extremely rapid-less than 15 seconds required to obtain an initial measurement on the spectrophotometer.

b. Stability:

In freshly prepared solution of Hb-SLS kept at room temperature no significant difference were seen in absorbance at 535nm on repeated measurements during the first 120 minutes after dilution (Figure 1).

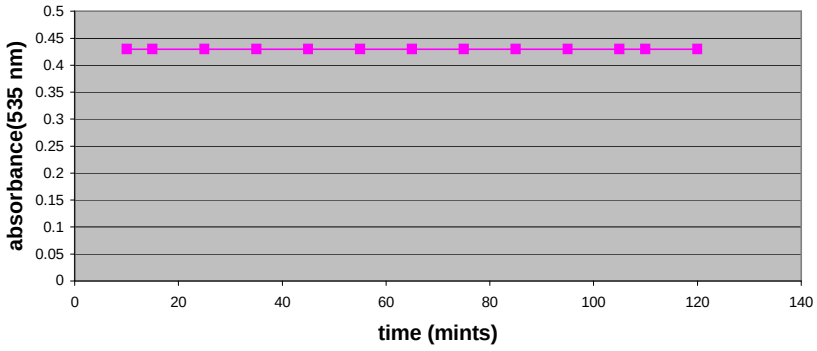

Figure 1: Haemoglobin SLS colorization

2. Technical Assessment:

a. Linearity:

Using a sample of fresh normal blood a series of 10 dilutions were made of packed red cells in platelet poor plasma. 2 replicate analyses were performed by spectrophotometer on each dilution and the mean results were plotted. (Table 1 and figure 2), with significant correlation (r = 0.999).

Table 1: The effect of dilution on measurement of SLS-Hb

Hb(g/l)	Slope	Intercept	Correlation coefficient(r)
SLS-Hb method	2.174	2.323	0.999

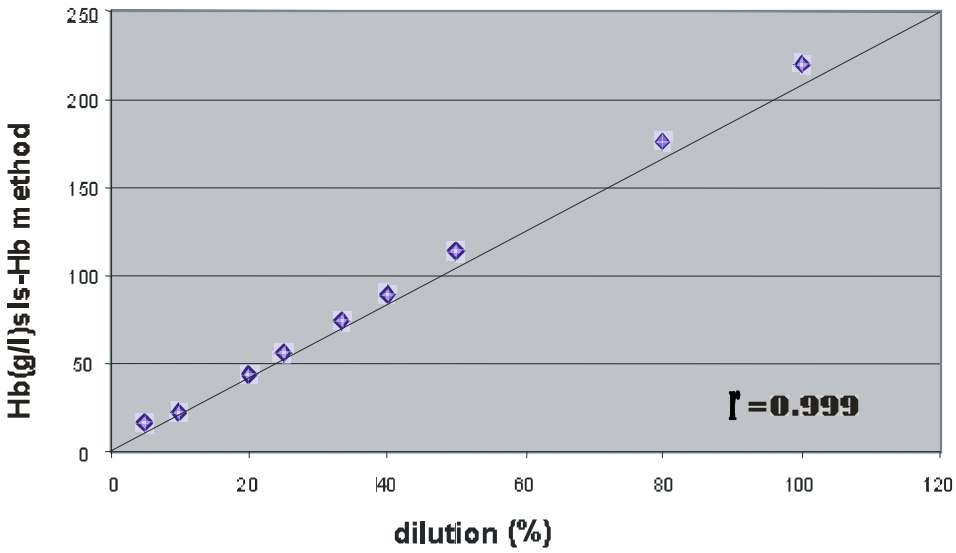

Figure 2: Showing the effect of dilution on measurement of SLS-Hb (linearity)

b. Comparability:

Results on 250 samples are shown in (Table 2 and figure. 3). Mean Hb (g/l) of 250 samples by SLS-Hb method was 116.9 ±31.05 (31-162), while the mean (g/l) by HiCN method was 116.84 ± 31.13 (31-162), with significant correlation (r = 0.998).

Table 2: Comparison of measurement of haemoglobin by SLS-Hb method and routine HiCN method

Method	**No. of samples**	***Mean /SD (g/l)***	**Min (g/l)**	**Max (g/l)**	**Correlation (r)**
Hb-SLS Method	250	116.9 ± 31.05	31	162	0.9983
HiCN Method	250	116.84 ± 31.13	31	162	

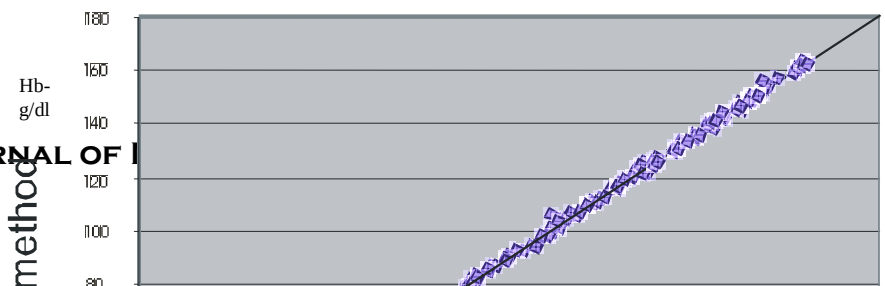

Figure 3: Comparison of measurement of haemoglobin by SLS-Hb method and routine HiCN

3. Interfering Substances:

a. Leukocytosis:

The effect of high leukocyte is illustrated in (Table 3 and figure 4). The differences from the reference method carried out in washed samples.

Table 3: The effect of WBC on measurement of Hb by SLS-Hb method and HiCN method

Method	No. of samples	Mean WBC count/cm	Hb(g/l) mean+SD	min (g/l)	max (g/l)	% Difference from Reference method (washed RBC)
SLS-Hb	12	63416.66	80.25±28.04	38	120	0.11%
HiCN	12	63416.66	80.66±27.81	39	120	0.62%
Hb after washed RBC	12	20583.33	80.16±30.51	37	125	

Note ;t = 0.890 p = 0.392 (non-significant)

b. Lipaemia:

The Hb on 15 selected samples were measured as SLS and as HiCN on whole blood and on spun plasma. Plasma reading were subtracted to obtain turbidity corrected measurements (Table 4).

C. Haemoglobin F:

Thirty-five specimens of neonatal (cord) blood were measured by SLS method and HiCN method, comparison of SLS and HiCN is show in (Table 5 and figure 5). Mean (g/l) of Hb by SLS method was 158.62 + 21.47 (110-187), while mean (g/l) by HICN was 158.85 + 59 (110-189), with significant correlation (r = 0.995).

4. Haemoglobin Derivatives:

Haemoglobin concentration was measured on blood samples by spectrophotometery as HiCN and Hb-SLS after converting proportion of Hb to methaemoglobin, (Table 6 and figure 6). Mean of Hb (g/l) by SLS method was 115.254+33.01 (36-163), while mean by HiCN was 115.08 + 32.74 (35-161), with significant correlation (r=0.999).

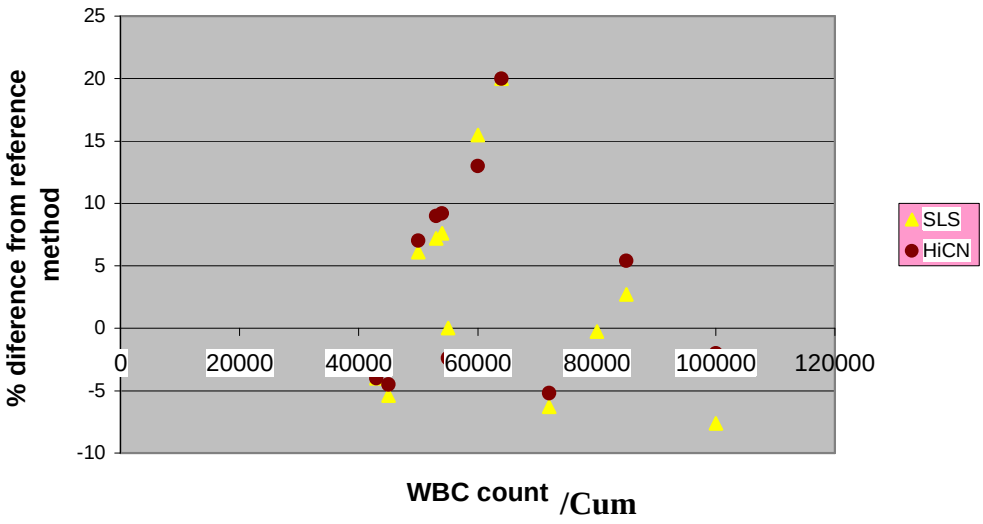

Figure 6: Effect of WBC on measurment of Hb by SLS-Hb method and HiCN method

Table 4: Mean ± SD of Hb (g/l) by SLS-Hb method and HiCN method on lipaemic samples pre and post correction

Samples	SLS – method (g/l)		HiCN method (g/l)	
	Direct	Corrected	Direct	Corrected
1	139	131	133	133
2	74	71	75	71
3	88	80	89	82
4	72	70	75	74
5	99	94	99	94
6	110	90	113	97
7	75	73	78	79
8	140	136	148	139
9	96	90	98	92
10	103	101	105	102
11	144	140	147	143
12	65	63	68	65
13	102	100	105	102
14	99	91	100	93
15	120	115	124	117
Mean±SD	101.73±25.35	96.26±24.62	103.8±25.44	98.86±24.49

Note; t-test for direct (SLS method) Vs direct (HiCN) = -2.77, p= 0.015 (significant)
T-test for direct Vs corrected = for (SLS) method t-test= -4.591, p=0.0001; for (HiCN) method t-test= 4.606, p= 0.0001

Table 5: The relationship between SLS-Hb method and HiCN method in measurement of cord blood (Hb F)

Method	No. of samples	Mean SD (g/l)	Min (g/l)	Max (g/l)	Correlation coefficient(r)
SLS-Hb method	33	158.62±21.47	110	187	r=0.9956,
HiCN method	33	158.85±21.59	110	189	p=0.0001

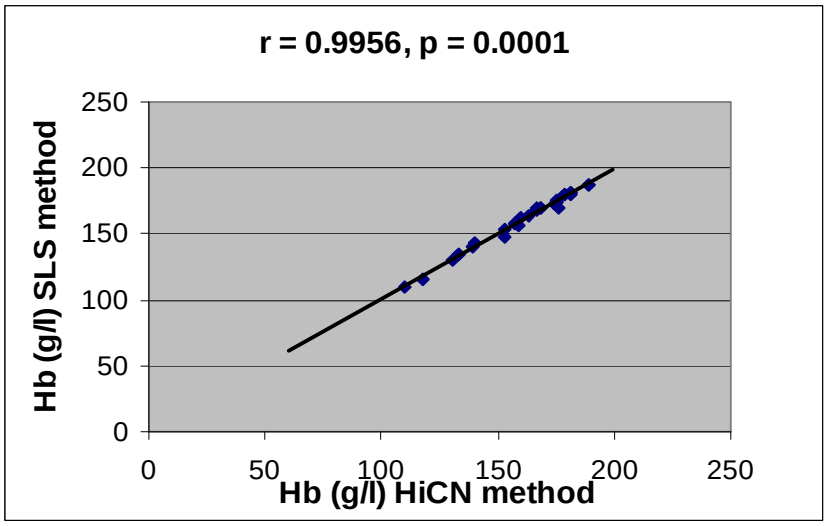

Figure 5: Comparison of measurement of HbF by HiCN method and SLS-Hb method

Table 6: The relationship between SLS-Hb method and HiCN method in measurement of methaemoglobin

Method	NO. of samples	Mean SD (g/l)	Min (g/l)	Max (g/l)	Correlation coefficient(r)
SLS-Hb	35	115.25±33.01	36	163	0.999
HiCN	35	115.08±32.74	35	161	

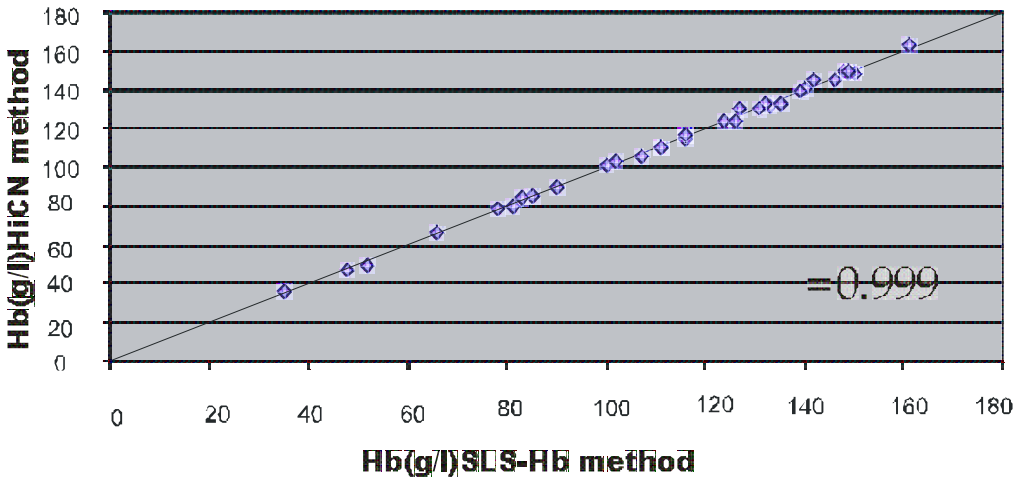

Figure 6: Comparison of measurement of methaemoglobin By SLS-Hb method and HiCN method

Discussion

The ideal method for haemoglobinometry should meet the following requirements:

1. Directly referable to a stable reference standard.
2. Immediate reaction between reagent and blood for total conversion of haemoglobin.
3. Perfect correlation between absorbance at an appropriate wavelength and haemoglobin concentration.
4. Stability of converted haemoglobin for at least several hours.
5. All haemoglobin derivatives are converted.

Reagent is non-toxic and does not affect the apparatus in which it is used in the ICSH recommended haemiglobincyanide (HiCN) method haemoglobin is converted by means of a ferricyanide reagent based on Drabkin's reagent[13]. This method, which has been universally accepted, meets the most important criteria of being linked with a stable standard, the international haemoglobincyanide reference standard[14]. This standard has a stability of at least six years.

The disadvantage of the HiCN method is that the reagent contains cyanide, and although this is used in a harmless amount for each assay, there is potential toxic risk where large volumes are discharged as waste: in some countries, this is regarded as a health hazard, which must be controlled in accordance with complex health and safety regulations. It is thus desirable to use a non-toxic alternative in routine practice[13].

Several alternatives are available[12], especially oxyhaemoglobin (HbO_2) using ammoniated water as reagent[15], but the product is unstable, and the method is unreliable in the presence of carboxyhemoglobin, methenoglobin, or sulfhemoglobin[16]. Nor do any other established methods meet the requirements set out above. It has been suggested that a better method might be to convert haemoglobin to a sulfate derivative by means of sodium Laury sulfate[11], this is a non-toxic substance[13]. It has now been developed as a commercial reagent by to a Medical Electronics for use in their automated haematology analysers and a preliminary report on its usefullness has been published[17]. This present study was undertaken to assess its use for haemoglobinometry in general.

The conversion time was extremely rapid less than 15 seconds. Lewis (1991) found the conversion time was less than 13

seconds[13], this was documented by this study. Similar results were also described by Karsan et al (1993)[11].

Repeated measurement of freshly prepared Hb-SLS during the first 120 mints after dilution shows no significant differences, (Figure 1). These findings were consistent with the findings of Lewis (1991) whose found that repeated measurements of freshly prepared solution of Hb-SLS during the first 2 hr, after dilution shows no significant differences.

Correlation coefficient (r) was 0.999 (Table 1and figure 2), in which there were some instrument differences, but no significant discrepancy for linearity. Lewis (1991) found that there was a direct relationship of absorbance to Hb concentration over awid range of measurements with (r) of 1.000[13].Similar results were encountered in this study.

Mean Hb (g/L) by SLS method was 116.9 $\pm$ 31.03 while mean Hb (g/L) by the HiCN method was 116.84 $\pm$ 31.13, with correlation coefficient of 0.998, (Table 2 and figure 3). Lewis (1991) found that there is a strong correlation between HiCN method and SLS method in measurement of Hb on routine blood specimens with a correlation coefficient of 0.998[13]. This was documented by this study. The same findings were also reported by Tsuda, Ntatsumi (1998) with correlation coefficient of 0.998[10]. The effect of high leucocyte count is illustrated in table 3 and figure4).

The differences from reference method carried out on washed red cells samples[18], showed no significant difference in measurement of Hb by SLS method and HiCN method. The findings of Lewis et al (1991) were not confirmed in this study, this may be due to the use of washed RBCs indicated the measurement of Hb by SLS method is less affected than HiCN method by the leucocytes[13].

The findings of Lewis (1991) did not confirmed by this study, this may be due to use washed RBC rather than centrifuged / filtered samples, because of unavailability of filters in Iraq.

Mean Hb (g/L) by SLS method was (101.73 $\pm$ 25.35 uncorrected and 96.26 $\pm$ 24.62 corrected), and by HiCN method was (103.8 $\pm$ 25.44 uncorrected and 98.86 $\pm$ 24.49 corrected) (Table 4). This findings was consistent with those of Lewis (1991) who founds that the SLS method was slightly more reliable on measurement of Hb when there's interference substance like lipaemia or WBC[13]. This was confirmed by this study.

Mean Hb (g/L) by SLS method was 158.62 $\pm$ 21.47 and by HiCN method was 158.85 $\pm$ 21.59, with correlation coefficient (r) of 0.995. There was no significant difference between SLS method and HiCN method on measurement on samples containing HbF (Table 5 and figure 5).

Lewis found that there was no difference on measurement of sample containing HbF by SLS and HiCN, with correlation coefficient of 0.993[13]. Similar results were encountered in this study.

Mean of Hb (g/L) by SLS method was 115.25$\pm$33.01 and by HiCN method was 115.08 $\pm$ 32.74 with correlation coefficient of 0.999 (Table 6 and figure 6). The findings of Lewis et al (1991) were confirmed in this study[13] in which there was no significant difference in measurement of methaemoglobin by SLS and HiCN methods[13].

Conclusions

The measurement of Hb by sodium lauryl sulfate showed:

1. It is non-toxic substance.
2. Hb converts almost instantaneously.
3. There is a direct relationship of absorbance to Hb concentration over a wide range of measurements.
4. It is stable for 2 hours without significant effect.
5. It is reliability is equal to that of haemoglobinocyanid method.
6. Slightly more reliable method when there is interference by lipaemia.
7. Measures Hb containing high concentration of HbF and methaemoglobin.

Recommendations

1. Haemoglobin sulphate (SLS) method provides a reliable routine method with the advantage of non-hazardous reagent.
2. We recommend another study using sodium lauryl sulfate in automated analysers.

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