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The Determination of Haemoglobin as Cyanhaemiglobin or as Alkaline Haematin D-575 Comparison of Method-Related Errors

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Summary: In order to compare the accuracy of haemoglobin (Hb) determination methods, the commonly used cyanhaemiglobin (HiCN) method and the recently developed alkaline haematin D-575 (AHD) method (*R. Zander, W. Lang & H. U. Wolf* (1984) Clin. Chim. Acta *136*, 83–93; *H. U. Wolf, W. Lang & R. Zander* (1984) Clin. Chim. Acta *136*, 95–104) were tested with respect to method-related errors such as plasma, cell, and Hb errors.

Both methods yield a series of more or less significant errors which generally lead to an overestimation of the Hb concentration in the order of 1%. However, in all three cases of plasma errors, i. e. normal plasma error, plasma error in lipaemic blood, and plasma error in bilirubinaemic blood, the AHD method shows significantly lower values of errors than the HiCN method.

In the case of cell errors such as ghost and leukocyte errors, the overestimation of the Hb concentration by the HiCN method is 60% higher than that by the AHD method. In the case of Hb errors such as fetal Hb and carboxy Hb errors, there is a significant overestimation of the Hb concentration by the HiCN method, which amounts 3 min after mixing of blood and HiCN solution to 0.7% in the case of fetal Hb and to 13.2% in the case of carboxy Hb. The latter value yields an overestimation of 1.3%, when 10% carboxy Hb in a blood sample is present. In contrast, there is no detectable overestimation after 3 min in the case of the AHD method.

Thus, the AHD method provides a higher accuracy in Hb determination than the commonly used HiCN method.

Introduction

A new method for the rapid and accurate measurement of haemoglobin has been developed as an alternative to the conventional HiCN method (1). This method is based on the conversion of all haem, haemoglobin, and haemiglobin species into a stable end product by an alkaline solution of a non-ionic detergent (e. g. Triton X-100). The reaction product, designated as alkaline haematin D-575 (AHD), is very stable and shows a characteristic absorption peak at 575 nm. As compared to the HiCN method, the determination of haemoglobin by the AHD method offers several advantages such as

(a) high stability of the AHD reagent and of the conversion product,

(b) decreased conversion time of all haemoglobin species into the end product, and

(c) the possibility of standardization with a primary standard, i. e. purified crystalline chlorohaemin (2).

As demonstrated by several authors (3 – 9), the HiCN method shows a series of different errors which can lead to an overestimation of the Hb concentration. Therefore, the two methods are compared with respect to plasma and cell errors, and errors caused by delayed conversion of carboxy and fetal Hb in the present paper.

Materials and Methods

Chemicals: Triton X-100, virtually peroxide-free, and NaOH were obtained from Merck, Darmstadt. Bilirubin was used as Dade® Bilirubin Control (containing albumin), from Dade Division, American Hospital Supply Corporation, Miami, USA.

Chlorohaemin was an own preparation according to a method described in detail in a special paper[1]).

Blood was heparinized human blood. The blood was treated with pure CO (Linde AG, Mainz-Kastel, FRG) to form carboxyhaemoglobin.

The HiCN standard reagent solution according to *Van Kampen & Zijlstra* was obtained from Merck, Darmstadt, and from Boehringer, Mannheim.

The AHD reaction solution consisted of 2.5% Triton X-100 and 0.1 mol/l NaOH (pH = 13).

The two methods were tested using 20 µl of blood or plasma + 5 ml HiCN reagent in the case of the HiCN method, and 20 µl of blood or plasma + 3 ml AHD reagent in the case of the AHD method, the absorbance being measured against the corresponding reagent solutions as blanks.

All photometer readings were run in the visible range at 575 and 540 nm, respectively, on a Zeiss precision photometer PM 6 (band width 2 nm, wavelength accuracy ± 0.5 nm).

The results for both methods listed in the tables are expressed as absorbance values (A) and haemoglobin concentration in g/dl. The latter values were obtained using equation (1)

$$[Hb]\ (g/dl) = 36.77 \cdot A_{540\,nm} \qquad \text{(Eq. 1)}$$

in the case of the HiCN method, and using equation (2)

$$[Hb]\ (g/dl) = 34.96 \cdot A_{575\,nm} \qquad \text{(Eq. 2)}$$

in the case of the AHD method.

The numerical factor in eq. (1) and eq. (2) is a conversion factor to obtain the Hb concentration in g/dl from absorbance values including the molecular weight of haemoglobin, the millimolar absorption coefficient and the dilution ratio of blood with respect to HiCN (14) and to chlorohaemin (2).

Results

Plasma errors

Normal plasma errors and its dependence on haematocrit value

The normal plasma error was measured using 14 samples of plasma from 8 persons. Each sample was determined 5 times (n = 70). The measured absorbance amounts to 0.0053 ± 0.0017 in the HiCN method and to 0.0027 ± 0.0011 in the AHD method. This normal plasma error obviously results in an overestimation of the Hb concentration, whereby both the absolute and relative degree of this overestimation depends on the haematocrit value. These calculated results are listed in table 1. At all Hb concentrations considered, the AHD procedure shows markedly lower positive errors than the reference HiCN method.

Plasma error in lipaemic blood

The plasma error in lipaemic blood was measured using 20 µl of lipaemic plasma from 2 test persons in 15 determinations. The absorbance value for the HiCN method was 0.0103 ± 0.0029, and for the AHD method 0.0074 ± 0.0043, and these are expressed in table 1 as Hb in g/dl. In addition, this table contains the positive relative errors calculated for lipaemic blood containing 15 g/dl (haematocrit = 0.45, plasma volume = 0.55 of the blood volume). Again the overestimation of Hb is lower in the case of the AHD method than with the reference method.

[1]) Chlorohaemin with a purity of > 99% can be obtained from Serva, Heidelberg FRG.

Tab. 1. Error of Hb determination caused by normal and pathological plasma

Kind of plasma	Haematocrit	Hb concentration [g/dl]	Residual plasma volume [µl]	Error in the Hb determination			
				HiCN method		AHD method	
				[g/dl]	[%]	[g/dl]	[%]
Normal	–	–	20	+0.195	–	+0.094	–
calculated influence of haematocrit	0.45	15	11	+0.11	+0.77	+0.052	+0.35
	0.30	10	14	+0.14	+1.47	+0.066	+0.45
	0.15	5	17	+0.17	+3.57	+0.080	+0.54
Lipaemic	0.45	15	11	+0.209	+1.4	+0.143	+0.95
Bilirubinaemic	0.45	15	11	+0.071	+0.47	+0.029	+0.19

Plasma error in bilirubinaemic blood

For the determination of this error, 20 µl of a freshly prepared bilirubin solution of 200 mg/l (n = 10) were used. The results, shown in table 1, are expressed as Hb in g/dl and as the relative Hb overestimation at 15 g/dl (haematocrit = 0.45, i.e. 0.55 of the whole blood volume is assumed to be plasma). In analogy to the plasma errors described before, the degree of overestimation again is lower in the AHD procedure.

Cell errors

Ghost error

An overestimation of Hb concentration is also caused by the fact that erythrocyte membranes are not dissolved completely by the Hb reagents used. This error was estimated as follows: Samples of 20 µl blood + 5 ml HiCN reagent or 20 µl blood + 3 ml AHD reagent were centrifuged for 60 min at 20 000 *g*. The sedimented membranes (possibly containing nuclei of leukocytes) were resuspended in 1/4 of the membrane-free supernatant. The absorbance of the membranes was determined by measuring the membrane suspension versus the membrane-free supernatant. The absorbance values obtained from five determinations were 0.0015 with the HiCN method and 0.0010 with the AHD method. The results are given in table 2 as the apparent Hb concentration, and as the relative error at 15 g/dl. In both methods the relative error due to ghosts is negligible and of no clinical importance. However, it is decreased by about a half in the new method.

Leukocyte error

Another overestimation of the Hb concentration is caused by the presence of leukocytes, which may not be completely dissolved in the reaction media of both Hb determination procedures. Leukocytes prepared from fresh blood were suspended in NaCl solution. The absorbance of 2 different dilutions (leukocyte count 31.6 and 116 $\times$ 10^9/l) were used to calculate the results given in table 2 on the basis of equations (1) and (2). The calculated values of the relative overestimation of the Hb concentration under physiological conditions (leukocyte count $5 \cdot 10^9$/l) and in leukocytosis (leukocyte count $50 \cdot 10^9$/l) indicate that the leukocyte error is considerably lower with the AHD method than with the HiCN method, and that, however, this error is markedly increased in the case of leukocytosis with both methods.

Tab. 2. Error of Hb determination caused by erythrocytes and leukocytes

Cells	Method	Apparent Hb concentration [g/dl]	Relative error at Hb concentration of 15 g/dl [%]
Erythrocyte ghosts	HiCN	0.055	+0.36
	AHD	0.035	+0.26
Leukocytes:			
Normocytosis ($5 \cdot 10^9$/l)	HiCN	0.024	+0.16
	AHD	0.015	+0.10
Leukocytosis ($50 \cdot 10^9$/l)	HiCN	0.24	+1.6
	AHD	0.15	+1.0

Hb errors

These errors arise because some Hb species or derivatives are converted into the reaction products considerably more slowly than normal Hb. Thus, measuring the absorbance of the sample prior to complete conversion may lead to wrong results, i.e., to an overestimation of the Hb concentration.

Fetal Hb

As compared to normal Hb, the time needed for conversion into HiCN is slightly increased in the case of fetal Hb. The time course of the conversion into HiCN, and into AHD, respectively, measured with 8 blood samples of 2 newborns, is shown in table 3, the results being expressed as the absorbance difference in percent of the value obtained after 60 min, this value being considered as the final absorbance value.

Tab. 3. Effect of conversion rate of fetal or carboxy Hb on Hb determination expressed as relative error in percent of the absorbance value at 60 min (fetal Hb) and at 120 min (carboxy Hb)

Hb species	Method	Time [min]								
		1	2	3	5	10	20	40	60	120
Fetal Hb	HiCN	+ 4.0	+ 1.6	+ 0.7	+ 0.4	+0.0	+0.0	+0.0	+0.0	+0.0
	AHD	+ 1.0	+ 0.0	+ 0.1	+ 0.1	+0.0	+0.0	+0.0	+0.0	+0.0
Carboxy Hb	HiCN	+16.7	+14.5	+13.2	+11.1	+8.4	+4.6	+3.4	+1.6	+0.0
	AHD	+ 1.3	+ 0.1	+ 0.1	+ 0.1	+0.1	+0.1	+0.0	+0.0	+0.0

From these results it is evident that the Hb concentration is overestimated by 0.7% when measured after 3 min. In contrast to these results there is practically no overestimation in the case of the AHD method, when measured after 2 min.

Carboxy Hb

It has been demonstrated by several authors (5–8) that the conversion of carboxy Hb into HiCN is significantly slower than that of carbonmonoxide-free Hb. The time-course of the conversion into the end-products is given for both methods in table 3. The results (n = 3 for each method) are expressed as the absorbance difference in percent of the value obtained after 120 min, this value being considered as the final absorbance value. There is a marked positive error of absorbance values in the case of the HiCN method during the first 20 min, in contrast to the values of the AHD method, in which the final absorbance value is obtained after 2 min. Obviously, this delayed conversion of carboxy Hb leads to an overestimation of the Hb concentration, whenever carboxy Hb is present in the blood. Assuming a carboxy Hb concentration of 10% of the total Hb concentration – a realistic value in blood of smokers (maximally 12–22% COHb, compare table 28 in l.c. (13)) –, a significant overestimation is obtained during the first 10 min with the HiCN method (tab. 4). The degree of overestimation is expressed as % of the total Hb concentration. In contrast to the conventional method, the method presented here does not result in an overestimation in normal blood (15 g/dl) after 2 min.

Tab. 4. Time dependence of the relative error on Hb determination in the presence of carboxy Hb. The values were calculated assuming a relative concentration of 10% carboxy Hb

Time difference between mixing of blood with corresponding Hb reagent and measurement [min]	Relative error in Hb concentration at 15 g/dl	
	HiCN method [%]	AHD method [%]
1	+1.7	+0.1
2	+1.5	+0.0
3	+1.3	+0.0
5	+1.1	+0.0
10	+0.8	+0.0

Discussion

The HiCN method overestimates the haemoglobin concentration as a result of errors caused by cells (4, 10), by normal or pathological plasma (4, 10–12), and by certain haemoglobin species (5–8). Our own studies confirmed the literature reports of these errors. As an example, *van Kampen* et al. (4) found a plasma error of + 0.4% (up to 3% in some cases of low Hb concentration and of highly coloured plasma) and an erythrocyte error of 0.2% (varying between 0 and 0.7%). These values are in good agreement with those found here: a plasma error in the range from 0.77% to 3.57% (tab. 1), and a ghost error of + 0.36% (tab. 2).

The corresponding values of the relative errors in the AHD method are significantly lower: normal plasma + 0.35%, lipaemic plasma + 0.95%, and ghosts + 0.26%. The error due to delayed conversion into the end product is zero, because conversion of all haemoglobin species including fetal and carboxy-Hb is complete even after 2 minutes.

Unfortunately, all errors described here are positive errors for both methods. Therefore, a considerable overestimation of the Hb concentration may arise, if several small positive errors occur at the same time. Two examples may be given to demonstrate the possible effects:

1. Hb determination for a smoker, measurement 3 min after the addition of the reaction solution, haematocrit 0.45, lipaemic plasma, normal red blood cells: The positive error (caused by plasma, lipaemia, ghosts, leukocytes, and 10% carboxy Hb) amounts to 4% for the HiCN and to only 1.6% for the AHD method.

2. Hb determination for a newborn, measurement 3 min after addition of the reaction solution, haematocrit 0.30, normal blood cells, [bilirubin] = 50 mg/dl: The positive error (caused by plasma, ghosts, leukocytes, bilirubin, and fetal Hb) amounts to 4.9% for the HiCN and to only 1.8% for the AHD method.

Turbidities caused by plasma proteins and by fragments of cells are a general problem and occur whenever haemoglobin is photometrically determined in blood, irrespective of which method may be used. The composition of the HiCN reagent is a compromise with respect to the pH value which is slightly above neutrality (pH = 7.2), accelerating the reaction on one side, but also favoring turbidities on the other (15). Normally, these difficulties can be minimized by the presence of a suitable detergent at a low concentration, e. g. 0.05 g/dl, which does not affect the HiCN spectrum. On the other hand, the AHD reagent, which is an aqueous alkaline (pH = 13) solution of a non-ionic detergent at a high concentration, e. g. 2.5 g/dl Triton X-100, causes rapid haemolysis of the blood with subsequent denaturation and oxidation of haemoglobin by aerobic oxygen dissolved in the reagent,

and almost complete solubilization of the constituents of blood by micellization. This may explain the relatively low methodological errors in the AHD procedure in comparison with the HiCN-method.

Although the errors of the HiCN method are generally not meaningful in practice, the results of the comparison of errors is another criterion by which the newly developed AHD method is superior to the reference method. Other advantages have been discussed in detail (1, 2), especially the possibility of direct standardization.

Addendum

One point which might become more important in the future has not yet been mentioned. It is well-known that cyanide is a detrimental hazard to the environment, e. g. to fish, on account of its high toxicity. This is one of the reasons why Japan has already restricted the use of cyanide. Indeed, this poison is delivered into the environment in considerable amounts especially when the HiCN method is run in automatic analysers. The hazardous effect of cyanide could be abolished by substituting the HiCN method by the AHD method. Triton X-100 is biologically degradable to at least 85%, and NaOH may be neutralized to NaCl prior to the release to the environment. NaCl is certainly not hazardous in quantities produced by the AHD method.

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