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Alkaline haematin D-575, a new tool for the determination of haemoglobin as an alternative to the cyanhaemiglobin method. I. Description of the method

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Summary

A new method for the rapid and accurate measurement of haemoglobin has been developed as an alternative to the conventional cyanhaemiglobin method. This method is based on the conversion of all haeme, haemoglobin, and haemiglobin species into a stable end product by an alkaline solution of a non-ionic detergent ('AHD reagent'). The reaction product, designated as alkaline haematin D-575, is extremely stable and shows a characteristic absorption peak at 575 nm. As compared to the cyanhaemiglobin method, the determination of haemoglobin by alkaline haematin D-575 offers several advantages such as (1) extreme stability of the AHD reagent and the conversion product, (2) decreased conversion time of all haemoglobin species into the end product, (3) decreased amounts of plasma and cell errors, and errors caused by delayed conversion of carboxy- and fetal haemoglobins, and (4) standardisation by a primary standard (purified crystalline chlorohaemin).

Introduction

Various methods for the quantitative determination of haemoglobin have been developed during the last decades, based on chemical or physical principles, gasometry, or spectrophotometry [1,2]. These methods differ markedly with respect to

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accuracy and precision [2–5]. Among these, the spectrophotometric method using cyanhaemiglobin has been accepted worldwide during 1953–1963 [6,7], and it has been recommended by the International Committee for Standardisation in Haematology (ICSH) in 1965 [8] and 1967 [9]. Since cyanhaemiglobin has been considered to be the only stable haemoglobin derivative [3,10], all other methods were judged to be rejected [5]. In Germany, this method has become a reference method, i.e., the use of other methods is only permitted, if the ratio of the results of the used method to those of the reference method is known [11].

Indeed, the cyanhaemiglobin method has — as compared to other known methods — several significant advantages: (1) only a single reaction solution has to be used; (2) all haemoglobin species and derivatives are monitored, i.e. deoxyhaemoglobin, oxyhaemoglobin, carboxyhaemoglobin, fetal haemoglobin, and (at least in part) sulphhaemoglobin; (3) the stable reaction product cyanhaemiglobin has a broad absorption maximum at 540 nm; (4) the Lambert-Beer law is valid within a wide range of absorption; (5) stable standard solutions can be prepared either from crystalline haemoglobin, or from washed erythrocytes, and they can be shipped without severe problems; and (6) since cyanhaemiglobin is a stable derivative of haemoglobin, measurements of the absorbance can be performed some minutes or even some days after the addition of blood to the reaction medium without significant alteration of the values obtained.

Nevertheless, the cyanhaemiglobin method has also some disadvantages (cf. [1], p. 740), which cannot be neglected: (1) since the cyanhaemiglobin reagent contains cyanide, it is toxic and therefore has to be handled very carefully; (2) the reaction solution is light-labile [10] (even in the dark, it is not stable for more than 6 months); (3) the concentrations of the reaction components, especially that of cyanide and the buffer have to be chosen and kept constant very carefully [12]; (4) standardisation of the method is based on purified cyanhaemiglobin solutions, the quality of which is controlled only indirectly by spectrophotometry (direct iron analysis is not possible because of the presence of $K_3[Fe(CN)_6]$ in these standard solutions); and (5) the reaction time of the different haemoglobin species and derivatives differ markedly, and at least in the case of carboxyhaemoglobin the time for conversion into cyanhaemiglobin seems to be too long for rapid clinical use, especially in automated haemoglobin analysis systems with a time period in the order of some 20 s. Under such conditions determination of haemoglobin as cyanhaemiglobin is not recommended.

The last disadvantage mentioned is a particularly crucial point. Originally, the conversion time had been determined to be 3 min for all haemoglobin species by Van Kampen and Zijlstra [10]. However, during the following years the conversion time for carboxyhaemoglobin into cyanhaemiglobin had to be corrected and was reestimated to be 90–120 min by Van Kampen and Zijlstra [13] and by Rodkey [14]. This period can be decreased to 15 min by increasing the ferricyanide concentration fivefold [15], or to 3–5 min by heating the reaction mixture to 56°C [16].

In order to circumvent these disadvantages of the cyanhaemiglobin method, we developed a new haemoglobin assay, which concomitantly should maintain the advantages and avoid the disadvantages of the conventional method mentioned

above. The result of this investigation is a haemoglobin method based on alkaline haematin, which by the addition of a non-ionic detergent changes significantly its properties concerning spectrum, formation velocity, and stability as compared to the original alkaline haematin described before [17–24]. One of the characteristic properties of alkaline haematin produced in the presence of non-ionic detergents is an absorbance peak at 575 nm. Therefore, this product was designated as 'alkaline haematin D-575' (D standing for detergent).

The present paper describes a haemoglobin assay on the basis of alkaline haematin D-575, the properties of that haematin species, and the precision and accuracy of the new method. A comparison with the standard cyanhaemiglobin method considering the errors made by these two methods will be published elsewhere.

Materials and methods

Chemicals: Triton X-100 (virtually peroxide-free), NaOH, KOH, $NaNO_2$, and sodium dodecylsulphate were obtained from Merck (Darmstadt, FRG), Brij 58 and benzyl-diisobutylphenoxy-ethoxyethyl-dimethyl-ammonium chloride were from Serva (Heidelberg, FRG), and dodecyl-dioxyethyl-benzyl-ammonium chloride was from Thomae (Biberach, FRG).

Bilirubin was from Dade® Bilirubin Control, Dade Division, American Hospital Supply Corporation (Miami, FL, USA). Cyanhaemiglobin standard solutions were obtained from Merck (Darmstadt, FRG) and Boehringer (Mannheim, FRG).

Chlorohaemin used in some of the experiments was our own preparation using a method described in detail in the subsequent paper (II. Standardisation of the method using pure chlorohaemin) [27].

Blood was heparinised human blood. It was equilibrated with an excess of purified nitrogen for the preparation of deoxyhaemoglobin, and untreated in the case of oxyhaemoglobin. The blood was treated with pure CO (Linde AG, Mainz-Kastel, FRG) to form carboxyhaemoglobin, and with H_2S to form sulphhaemoglobin. Haemiglobin was prepared by the addition of solid $NaNO_2$ to a final concentration of 0.1–0.2%.

All photometer readings were run in the visible range at 575 and 540 nm, respectively, using a Zeiss precision photometer PM 6 and a Perkin-Elmer photometer 550 S.

Results and discussion

Properties of the alkaline haematin D-575 reagent; haemolysis

The general procedure for haemoglobin determination by the alkaline haematin D-575 method is performed by mixing 20 μl of blood with 3 ml of a solution of 25 g Triton X-100 in 1 l of 0.1 mol/l NaOH. This solution is referred to as 'AHD reagent'. It can be handled easily, it is light-insensitive and stable for more than 1 year. Compared to the cyanhaemiglobin (HiCN) reagent used in the cyanhaemiglobin method containing cyanide, the new AHD reagent is considerably less toxic.

After mixing blood with AHD reagent, haemolysis occurs immediately and is virtually completed after few seconds. The olive-green solution does not show any turbidity or precipitation, even in blood samples with an increased amount of lipid. Erythrocyte ghosts are apparently solubilised almost completely. Practically the same results are obtained, when the Triton X-100 concentration is varied between 10 and 50 g/l or when the NaOH concentration is varied between 0.01 and 1.0 mol/l, or when Triton X-100 is replaced by other non-ionic detergents such as Brij 58.

Furthermore, the absorbance of the plasma is lower when diluted with the AHD reagent as compared to the dilution with HiCN reagent, since lipid material in the plasma is dispersed by the action of the non-ionic detergent. Consequently, the use of the AHD reagent leads to a decrease of cell and plasma errors as compared to the common cyanhaemiglobin method (see below).

General properties of alkaline haematin D-575

Alkaline haematin D-575 differs from the well-known alkaline haematin, which is obtained from haemoglobin in the presence of diluted alkali, but in the absence of detergents, with respect to three important properties: (1) spectrum, (2) velocity of formation, and (3) stability.

(1) In contrast to common alkaline haematin [19,21,22], alkaline haematin D-575 generated by means of the AHD reagent shows a distinct absorption peak at 575 nm, and a shoulder around 600 nm (Fig. 1). This spectral behaviour is produced not only by Triton X-100, but also by other non-ionic detergents such as Brij 58 (data not shown). As outlined above, this product will therefore be designated 'alkaline haematin D-575' in future.

In 1933, Heilmeyer [25] described the spectrum of alkaline haematin as highly dependent on the solvent. Therefore, it is somehow surprising that the spectrum of alkaline haematin is very similar in the presence of Triton X-100 and Brij 58, although these compounds have rather different structures [26]. Thus, the effects of non-ionic detergents on alkaline haematin seem to be rather unspecific.

The pattern of the spectrum of alkaline haematin D-575 is not influenced by the type of the starting material, e.g. chlorohaemin, fresh human blood, or cyanhaemiglobin. As shown in Fig. 2, in all cases nearly identical absorbance spectra are obtained. This is also true for other haemoglobin species or derivatives such as carboxyhaemoglobin, sulphhaemoglobin, or fetal haemoglobin.

This result is an absolute prerequisite for a haemoglobin method, the results of which are expected not to depend on the relative amounts of the different haemoglobin species present in blood.

(2) The rate of formation of alkaline haematin D-575 is markedly increased as compared to alkaline haematin, since the reaction is completed at optimum concentrations of NaOH and Triton X-100 present in the AHD reagent within 1–2 min at room temperature. The time necessary for complete conversion was found experimentally (data not included) to be the same for all haemoglobin, haemiglobin, and haeme species, i.e. not only for oxy- and deoxyhaemoglobin, but also for fetal haemoglobin, sulphhaemoglobin, haemiglobin, and even cyanhaemiglobin regarded as extremely stable, and last but not least for haeme and haemin species, e.g.

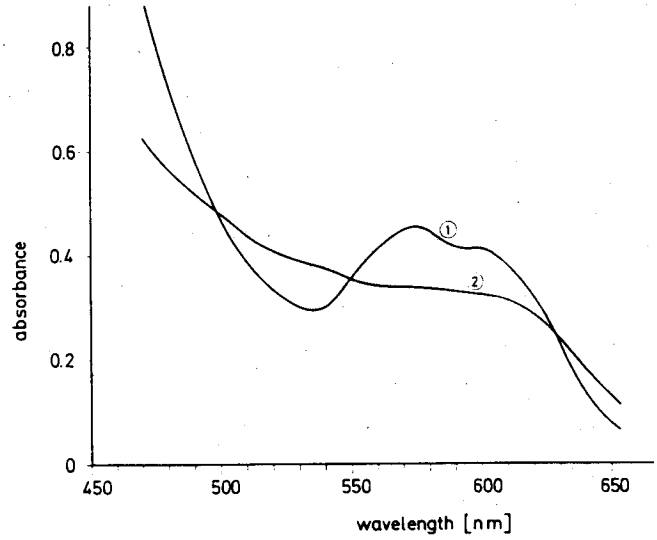

Fig. 1. Comparison of the spectra of alkaline haematin D-575 and alkaline haematin. Curve 1: spectrum of alkaline haematin D-575 produced by mixing a cyanhaemiglobin standard solution (212.4 mg cyanhaemiglobin/100 ml) with the same volume of double concentrated AHD reagent. Curve 2: spectrum of alkaline haematin produced by mixing this cyanhaemiglobin standard solution with the same volume of 0.2 mol/l NaOH.

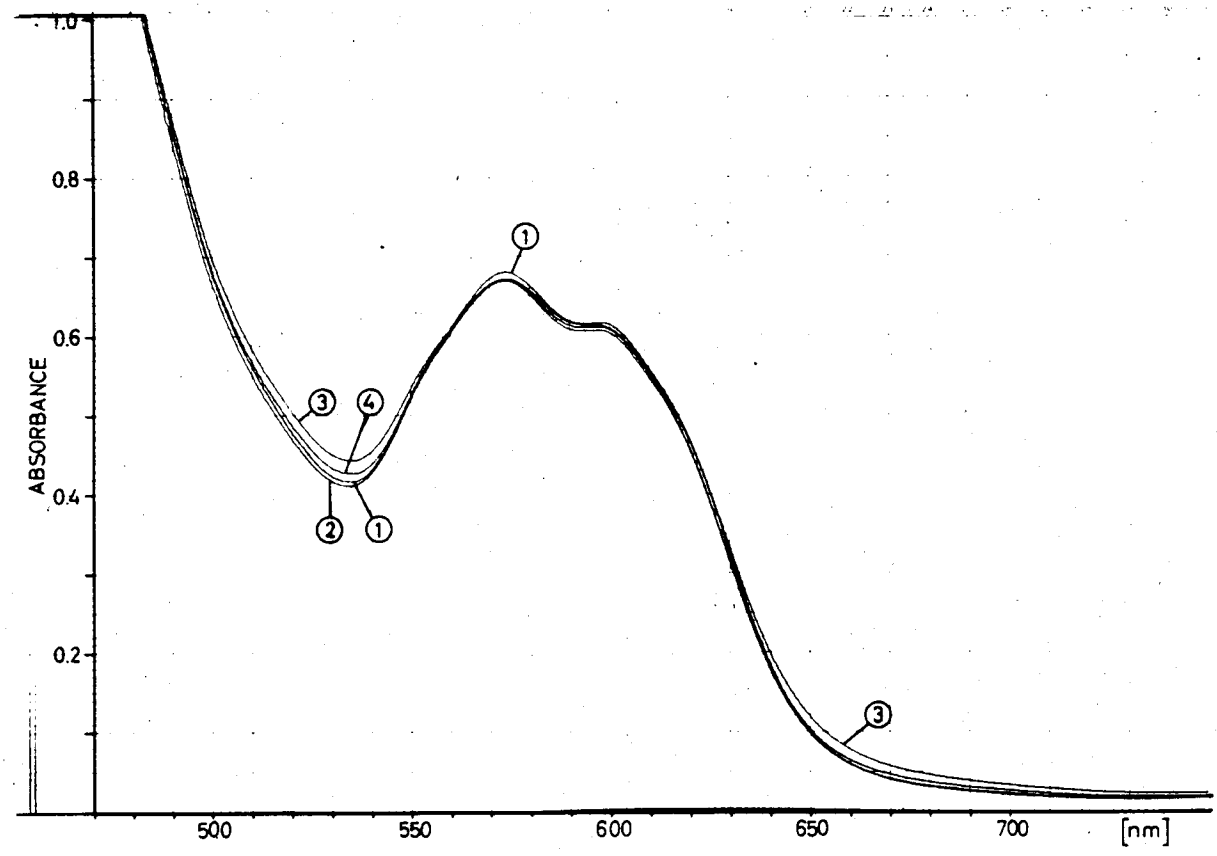

Fig. 2. Comparison of absorption spectra of alkaline haematin D-575 produced from different starting materials. Curve 1: from purely prepared chlorohaemin dissolved in AHD reagent (0.1 mmol/l) after 1 year storage in a brown bottle in a refrigerator. Curve 2: from chlorohaemin solution of the same concentration as curve 1, but freshly prepared. Curve 3: from freshly drawn blood, which was concentrated to a c_{Hb} of about 24.4 g/100 ml by removal of plasma, and treated with AHD reagent using the alkaline haematin D-575 procedure. Curve 4: from a concentrated cyanhaemiglobin reference solution (210 mg/100 ml), which was adjusted by dilution with a concentrated AHD reagent to a value corresponding to a c_{Hb} of 23.78 g/100 ml.

chlorohaemin. Thus, measurement of haemoglobin is possible as soon as 2 min after mixing blood and AHD reagent independently of the haemoglobin fractions present in blood.

(3) In contrast to alkaline haematin, which does not form a stable and well-defined product [4], pure alkaline haematin D-575 is a highly stable compound. The absorbance of blood, diluted with the AHD reagent does not change significantly within a period of several days, which is in any case a period sufficiently long for clinical practice.

Standard solutions of purified chlorohaemin, dissolved in the AHD reagent, could be stored in brown bottles at 4°C in the refrigerator or even at room temperature without changing absorbance by more than $\pm 0.5\%$ within a period of 2 years (see subsequent paper [27]).

Most important for the practical application of this method is the fact that identical results with respect to spectrum and stability are obtained when the concentration of Triton X-100 is varied in the range of 10–50 g/l, or when the NaOH concentration is varied in the range of 0.01–1.0 mol/l, or when Triton X-100 is replaced by other non-ionic detergents such as Brij 58, or when NaOH is replaced by other alkaline reagents such as KOH. In addition, any combination of these variations leads to the same result.

A decrease in the reaction rate of the formation of alkaline haematin D-575 is only observed when the applied concentrations of Triton X-100 and/or NaOH deviate from the above defined range of optimum concentrations.

This provides high stability of the system against incidental changes in the composition of the AHD reagent.

However, the method does not work, when the non-ionic detergents are replaced by cationic detergents such as benzyl-diisobutylphenoxyethoxy-ethyl-dimethylammonium chloride and dodecyl-dioxyethyl-benzyl-ammonium chloride, or by anionic detergents such as sodium dodecylsulphate.

Linearity of absorbance

In experiments, which are described and discussed in detail in the subsequent paper [27], a strong linearity between the concentration of purified chlorohaemin dissolved by AHD reagent and the absorbance could be demonstrated. The concentration range of chlorohaemin tested was equivalent to the clinical range of 5–25 g haemoglobin/100 ml.

Comparison of the method with the standard cyanhaemiglobin method

For a direct comparison of the two methods, blood of 25 persons (19 non-smokers and 6 smokers) was adjusted after centrifugation (by removal of plasma or erythrocytes) to three different haemoglobin concentrations between 5.9 g/100 ml and 24.2 g/100 ml. A sample of 20 μl blood was mixed thoroughly with 5 ml HiCN reagent in the case of the cyanhaemiglobin method and 3 ml AHD reagent in the case of the alkaline haematin D-575 method, respectively. The absorbance values were read 3 and 15 min after the addition of blood in a flow-through cuvette with 1 cm light path. These results finally had to be converted into haemoglobin concentrations.

In the cyanhaemiglobin procedure, the haemoglobin concentration of the blood sample in g/100 ml blood (c_{Hb}) was calculated on the basis of cyanhaemiglobin as a reference standard from the measured absorbance at 540 nm, ΔE_{540}, by the well-known equation:

$$c_{Hb} = 36.77 \cdot \Delta E_{540} \quad (1)$$

An analogous equation, based on the millimolar absorbance coefficient of standard chlorohaemin reference solutions (see subsequent paper [27]) was used in the case of the alkaline haematin D-575 procedure. With ΔE_{575}, the measured absorbance at 575 nm, the total haemoglobin concentration was calculated as

$$c_{Hb} = 34.96 \cdot \Delta E_{575} \quad (2)$$

All 75 determinations (25 test persons, 3 c_{Hb}) were made 3 times, and the mean values were used for the comparison of the two methods.

Accuracy. After plotting the results, i.e. the c_{Hb} values obtained, of the cyanhaemiglobin method (abscissa) versus the results of the alkaline haematin D-575 method (ordinate), straight lines are obtained, independently whether this is done for the group of (non-smokers + smokers), for the group of non-smokers, and for the group of smokers (figures not shown), and independently whether the measurement was performed after 3 or 15 min. These regression lines can be described by four slightly different equations, which are given in Table I. These equations show both a very slight deviation from the theoretical slope of 1.000 and from the theoretical intersection point on the ordinate of ± 0.000. The correlation coefficient is better than 0.988 in all cases.

Using these equations, the haemoglobin concentrations to be measured by the alkaline haematin D-575 method ($= y$) could be calculated by taking haemoglobin concentrations in the range from 5.0 to 25.0 g/100 ml for the cyanhaemiglobin reference method ($= x$), e.g. a physiological value of 15.0 g/100 ml. The results of these calculations are shown in Table II. From the data of the two Tables the following conclusions may be drawn.

TABLE I

Equations for the regression lines derived from the comparison of the alkaline haematin D-575 method (y) with the reference method (x)

Group of test persons	No. of test persons	Reaction time (min)	No. of measurements	Calculated regression line	Correlation coefficient
Non-smokers + smokers	25	15	75	$y = 1.005x + 0.004$	0.996
Non-smokers	19	15	57	$y = 1.021x - 0.151$	0.993
Smokers	6	15	18	$y = 0.978x + 0.222$	0.993
Smokers	6	3	18	$y = 0.970x + 0.321$	0.989

TABLE II

Haemoglobin concentration for different test groups, calculated with the equations for the regression lines from Table I, assuming a physiological haemoglobin concentration of 15 g/100 ml for the cyanhaemiglobin reference method

Group of test persons	Reaction time (min)	c_{Hb} (g haemoglobin/100 ml)
Non-smokers + smokers	15	15.08
Non-smokers	15	15.17
Smokers	15	14.89
Smokers	3	14.86

(1) Considering the results obtained after a reaction time of 15 min in the case of non-smokers + smokers, there is generally a difference of 0.5% in the experimental value, the alkaline haematin D-575 method yielding higher values than the reference method in the range of 5.9 g and 24.2 g haemoglobin/100 ml (Table I). At the moment, there is no indication whether the reference method underestimates the true haemoglobin content. The difference between the methods is higher in the case of the smokers, when the reaction time is limited to 3 min (Table I, lower line).

(2) There are slight, but not significant differences between the values for smokers and for non-smokers in the normal range around 15 g haemoglobin/100 ml, the deviation not exceeding ± 1% (Table II). The alkaline haematin D-575 method yields lower results for smokers and higher results for non-smokers than the reference method. This phenomenon can be interpreted as an overestimation of the haemoglobin content by the cyanhaemiglobin method, since the conversion of carboxyhaemoglobin into cyanhaemiglobin needs considerably more time than the conversion into alkaline haematin D-575 (data not included). This effect is still increased, when the reaction time is lowered to 3 min.

Precision. As shown in Table III, the mean value of all standard deviations of the 75 determinations and the relative mean deviation taken at 15.0 g haemoglobin/100 ml is nearly identical for both methods.

Precision from day to day. In order to evaluate the precision of the alkaline haematin D-575 method from day to day, a blood sample was used which had been

TABLE III

Comparison of the mean value of the standard deviations and the relative mean deviation of the two haemoglobin determination methods

Method	Mean value of SD (g haemoglobin/ 100 ml)	Relative mean deviation at 15 g haemoglobin/100 ml (%)
Cyanhaemiglobin method ($n = 75$)	±0.094	±0.63
Alkaline haematin D-575 method ($n = 75$)	±0.097	±0.65

prepared from an erythrocyte concentrate by dilution with 0.9% NaCl to a haematocrit of 47% under sterile conditions. The haemoglobin content of this sample was determined on 13 different days during a period of 40 days. Ten determinations using 20 μl of blood were made each day. The results of these determinations are presented in Table IV. The standard deviation of the 13 mean values (obtained on 13 different days) is 0.67% of the measured value, the mean and standard deviation of all 130 values being 14.88 $\pm$ 0.14 (= 0.94%) g haemoglobin/100 ml.

These values indicate that there is practically no difference in the accuracy and precision of the two methods, and that the alkaline haematin D-575 method yields results which do not differ significantly from day to day over a long period. These findings are in marked contrast to the results obtained with the haemoglobin method based on normal alkaline haematin, which has been considered not to be useful for the determination of haemoglobin [4,5,18,23].

Comparison of errors

It is well known that the cyanhaemiglobin method shows a series of different errors, which can lead to an overestimation of the haemoglobin concentration [5,10,13–15,25]. The degree of these errors, mainly plasma, cell, and haemoglobin errors, was tested for the two methods using 20 μl of blood or plasma + 5 ml HiCN reagent in the cyanhaemiglobin method, and 20 μl blood or plasma + 3 ml AHD reagent in the alkaline haematin D-575 method, measured against the corresponding reagent solution as blanks. The comparison of the results of the two methods showed clearly that in the case of the alkaline haematin D-575 method all errors were

TABLE IV

Precision from day to day of the alkaline haematin D-575 method

Day of measurement	c_{Hb} (g/100 ml)	
	mean (n = 10)	$\pm$ SD
1	14.88	0.07
2	14.59	0.06
3	14.98	0.05
8	14.84	0.12
9	14.92	0.15
11	14.79	0.10
17	14.97	0.09
23	14.93	0.06
26	14.97	0.08
36	14.88	0.09
38	14.91	0.08
39	14.86	0.07
40	14.83	0.12
Mean and standard deviation of the 13 mean values:	14.87 $\pm$ 0.10 ($\pm$ 0.67%)	

smaller than in the case of the cyanhaemiglobin method. The detailed experimental results of this comparison will be published in a separate paper.

On the basis of the data presented in this paper, it may be concluded, that the alkaline haematin D-575 method for haemoglobin determination offers some convincing advantages over the cyanhaemiglobin reference method, combined with a lack of any significant disadvantages.

These advantages are: (1) both the AHD reagent itself and the alkaline haematin D-575 formed by that haemoglobin reagent are extremely stable; (2) all haemoglobin species with clinical significance including fetal haemoglobin, sulphhaemoglobin, and carboxyhaemoglobin, and the derivative chlorohaemin are converted into one single end product, and can be monitored in that form easily; (3) in comparison to the reference method, the amounts of plasma and cell errors, and errors caused by delayed conversion of carboxy- and fetal haemoglobin into the monitored end product are decreased; (4) since Triton X-100 and NaOH are rather cheap chemicals, the method can be run routinely without high expenses; and (5) the method is based on a reaction medium not containing cyanide, a compound with high toxicity.

However, the most meaningful advantage of the method presented here is the fact that this method can be standardised by a solid standard, which is stable in crystalline and even in dissolved form, which is a well-defined compound, which is converted into the same end products as all haemoglobin species of clinical interest, and which can be handled easily for weighing, because it is e.g. not hygroscopic. This compound is chlorohaemin, which can be obtained now in a very pure form, and which can be used to standardise the alkaline haematin D-575 method with success. The possibility of a direct standardisation, which is described in the following paper [27], and the fact of a shorter conversion time for all haemoglobin species of clinical importance are strong arguments in favour of the use of the alkaline haematin D-575 method rather than the common cyanhaemiglobin method, which can only be standardised indirectly by adjusting standard cyanhaemiglobin solutions spectrophotometrically.

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