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Alkaline haematin D-575, a new tool for the determination of haemoglobin as an alternative to the cyanhaemiglobin method. II. Standardisation of the method using pure chlorohaemin

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Summary

Chlorohaemin with high purity (> 99%), a stable and well-defined compound, can be used as a primary standard for the standardisation of a haemoglobin assay based on alkaline haematin D-575 [6]. Dissolved in a solution of 25 g Triton X-100 per litre of 0.1 mol/l NaOH ('AHD solution'), the millimolar absorbance coefficient of the end product (alkaline haematin D-575) is 6.960 ± 0.046 [$l \cdot mmol^{-1} \cdot cm^{-1}$] at 575 nm. Within the range of haemoglobin concentrations of 5 to 25 g/100 ml there is a strong linear relation between chlorohaemin concentration and absorbance with a deviation of ≤ 2% from the theoretical values. As compared to the conventional cyanhaemiglobin standard solutions, standardisation with pure chlorohaemin is the method of choice because of the simplicity of the preparation of standard solutions, which can be done in every laboratory, and the stability of both the solid compound chlorohaemin and its solutions in alkaline Triton X-100.

For the first time a real standard for quality control in haemoglobinometry is recommended: a concentrated solution which behaves like blood, i.e. the simulation of all steps in haemoglobin determination (dilution and photometry) is possible.

Introduction

For the determination of haemoglobin, the spectrophotometric method using cyanhaemiglobin has been accepted worldwide [1,2] and recommended by the

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International Committee for Standardisation in Haematology (ICSH) in 1965 [3] and 1967 [4]. Despite its advantages, it has at least one disadvantage: the production of standard solutions is circumstantial and time-consuming. The standard solutions consisting of cyanhaemiglobin have to be prepared, then shipped to the national standardising organisations (e.g. in the FRG the Institute for Standardisation and Documentation at Freiburg/Breisgau), analysed only indirectly by spectrophotometry for their exact content, shipped back to the producing company and diluted in an appropriate way to the final concentration used as standard. Although there are quite a lot of different types of standard solutions (c.f. Von Klein-Wisenberg [5]), none of them is satisfactory, not the least because of the instability of the haemoglobin and cyanhaemiglobin solutions. Despite the considerable content of cyanide in these solutions, surprisingly they are not stable against bacterial infections.

In the meantime, cyanhaemiglobin has been accepted by the World Health Organisation as a secondary biological standard. Nevertheless, the same institution recommended strongly the development of a synthetic standard with absorbance characteristics very similar to or identical with those of cyanhaemiglobin [5]. This aim apparently has not been reached.

In a previous paper [6], a new method was described for haemoglobin determination based on alkaline haematin D-575. It could be demonstrated that this method has several advantages over the commonly used cyanhaemiglobin method. The most important is the possibility of standardisation by a primary standard which could cancel out the severe disadvantages of the cyanhaemiglobin method: the dependence on special standardisation solutions prepared commercially and analysed in special laboratories.

As reported in the previous paper [6], chlorohaemin as well as all haemoglobin species and derivatives are transformed by the alkaline solution of Triton X-100 ('AHD-reagent' = alkaline haematin detergent reagent) into a stable and spectrophotometrically defined end product, designated 'alkaline haematin D-575'. Thus, chlorohaemin, a well-defined and stable compound, could serve as a primary standard for the haemoglobin method described. However, the chlorohaemin preparations commercially available are generally insufficiently pure for use as a primary standard. In contrast, a modified procedure of the method of Labbe and Nishida [7] increased the purity of the chlorohaemin to practically 100%, which satisfies the requirements for a primary standard (see preceding paper [6]).

Furthermore, for laboratory use in clinical chemistry an optimal quality control system should be able to control not only the photometry (cuvette, band width, wavelength, etc.), but also all other steps in the analysis (e.g., dilution of blood).

In the case of haemoglobinometry a standard solution with the same behaviour as human blood would be desirable. Then both the dilution procedure and the photometric measurement could be controlled, and all errors in dilution and photometry could be excluded.

The present paper describes the standardisation of the alkaline haematin D-575 method by cyanhaemiglobin solutions and by purified chlorohaemin as a primary standard and an optimal quality control.

Materials and methods

Chemicals

NaOH, $SrCl_2 \cdot 6\ H_2O$, ethanol, diethylether, pyridine, 96% acetic acid (all p.a.) and acetone (pract.) were from Merck (Darmstadt, FRG). Triton X-100 (pure), used for the preparation of a solution of 25 g Triton X-100 in 1 l 0.1 mol/l NaOH referred to as 'AHD reagent' in the following, was obtained from Serva (Heidelberg, FRG). However, instability and scattering of the alkaline haematin D-575 absorbance values, possibly due to a varying content of peroxides in different Triton X-100 lots, prompted us to use only purified material. AHD reagents prepared with this material yielded identical alkaline haematin D-575 absorbance values for several weeks, when prepared some days prior to use. Exposure to light during storage of these reagents had no detectable influence on the absorbance data of alkaline haematin D-575.

Commercial chlorohaemin was from Fluka (Buchs, Switzerland), from Aldrich (Nettetal, FRG), from Koch-Light (Colnbrook, Bucks, UK), from Roth (Karlsruhe, FRG), or from Serva (Heidelberg). Cyanhaemiglobin (HiCN) standard solutions were obtained from Merck (Darmstadt, FRG) and Boehringer (Mannheim, FRG). Water was Ampuwa® water from Fresenius (Wiesbaden, FRG).

Blood was human blood obtained from Deutsches Rotes Kreuz, Ulm/Donau, FRG.

Preparation of pure chlorohaemin

Pure chlorohaemin was prepared essentially by the method of Labbe and Nishida [7] with a series of modifications. The complete procedure including recrystallisation, and the properties of the purified chlorohaemin, which shows a purity of > 99% according to the elemental analysis, will be published in detail in a separate paper.

Chlorohaemin solutions

In the experimental section, the following chlorohaemin solutions were used ($M_r = 651.95$, expected absorbance at 575 nm in AHD reagent corresponding to a millimolar absorbance coefficient of 6.960 $l \cdot mmol^{-1} \cdot cm^{-1}$):

Preparation procedure	Concentration [mmol/l]	Dilution	Expected absorbance at 575 nm
20 mg/500 ml	0.06135	none	0.427
65.2 mg/l	0.100	none	0.696
50 mg/500 ml	0.1534	none	1.068
65.2 mg/10 ml	10.0	1:151	0.461

Measurement of absorbance

The measurement of absorbance was carried out by two different photometers: spectral photometer PM 6 (Zeiss, Oberkochen, FRG) and a Perkin-Elmer UV/VIS photometer 550 S.

Results and discussion

Evaluation of the millimolar absorbance coefficient of chlorohaemin in the AHD reagent

For the evaluation of the millimolar absorbance coefficient of chlorohaemin in the AHD reagent at $\lambda = 575$ nm, six different chlorohaemin preparations of our own and six recrystallised samples of commercial crude chlorohaemin were used. From each of the 12 samples a solution of 20 mg in 500 ml of AHD reagent was prepared. The AHD reagent used for these solutions had been matured at least for 3 days. Each of the 12 samples was measured on 2 different days during a period of 8 days. The mean value of the absorbance measurements of the first day was

$$\Delta E_{(\lambda=575\,\mathrm{nm})} = 0.4268 \pm 0.0032 \; (n = 12) \tag{1}$$

and the mean of the measurements of the second day was

$$\Delta E_{(\lambda=575\,\mathrm{nm})} = 0.4273 \pm 0.0025 \; (n = 12) \tag{2}$$

Evidently, there is no time dependence in the absorbance values of the 12 chlorohaemin samples over that period. Hence, the mean value of all 24 absorbance measurements was

$$\Delta E_{(\lambda=575\,\mathrm{nm})} = 0.4270 \pm 0.0028 \; (n = 24) \tag{3}$$

The millimolar absorbance coefficient of chlorohaemin is obtained by dividing this value by the concentration of chlorohaemin used, which was 0.06135 mmol/l. Hence, the millimolar absorbance coefficient of chlorohaemin in the AHD reagent at $\lambda = 575$ nm is

$$\epsilon_{\mathrm{mmol}} = 6.960 \pm 0.046 \; [\mathrm{l} \cdot \mathrm{mmol}^{-1} \cdot \mathrm{cm}^{-1}] \tag{4}$$

Stability of the absorbance values of chlorohaemin in the AHD reagent

Solutions of chlorohaemin of various concentrations in the AHD reagent show high stability of the absorbance at $\lambda = 575$ nm at least for several months, when the Triton X-100 used for the preparation of this reagent is virtually free of peroxides (see 'Materials and methods'). In a series of experiments, which were started in June 1978, a 0.1 mmol/l solution of chlorohaemin was stored in brown bottles at 4°C and at room temperature. The absorbance of this chlorohaemin solution remained constant without any significant deviation for at least 2 years (data not included).

In contrast, whenever the presence of peroxides could be demonstrated, e.g. by the method of Lever [8], the absorbance dropped significantly especially during the first hours after dissolution of chlorohaemin and did not become stable during the following days, in some cases even not during the following weeks.

Validity of Lambert-Beer's law for alkaline haematin D-575 solutions

The test for linearity between the amount of chlorohaemin dissolved in the AHD

TABLE I

Experimental and calculated absorbance values of diluted and undiluted stock solutions of 50 mg chlorohaemin in 500 ml of AHD reagent

Dilution ratio	Absorbance [ΔE]		$\frac{\Delta E_{experim.}}{\Delta E_{calcul.}}$
	experimental	calculated	
20/100	0.218	0.214	1.021
40/100	0.422	0.427	0.988
60/100	0.637	0.641	0.995
80/100	0.845	0.854	0.989
100/100	1.051	1.068	0.985

reagent and the absorbance of the alkaline haematin D-575 originated from chlorohaemin was achieved in two different ways.

In one series of experiments, 50 mg of chlorohaemin were dissolved in 500 ml of the AHD reagent to yield a concentration of 0.1534 mmol/l. This stock solution was diluted in the ratios 20/100, 40/100, 60/100, and 80/100. Absorbance measurements of these four dilutions and the undiluted solution yielded values which slightly

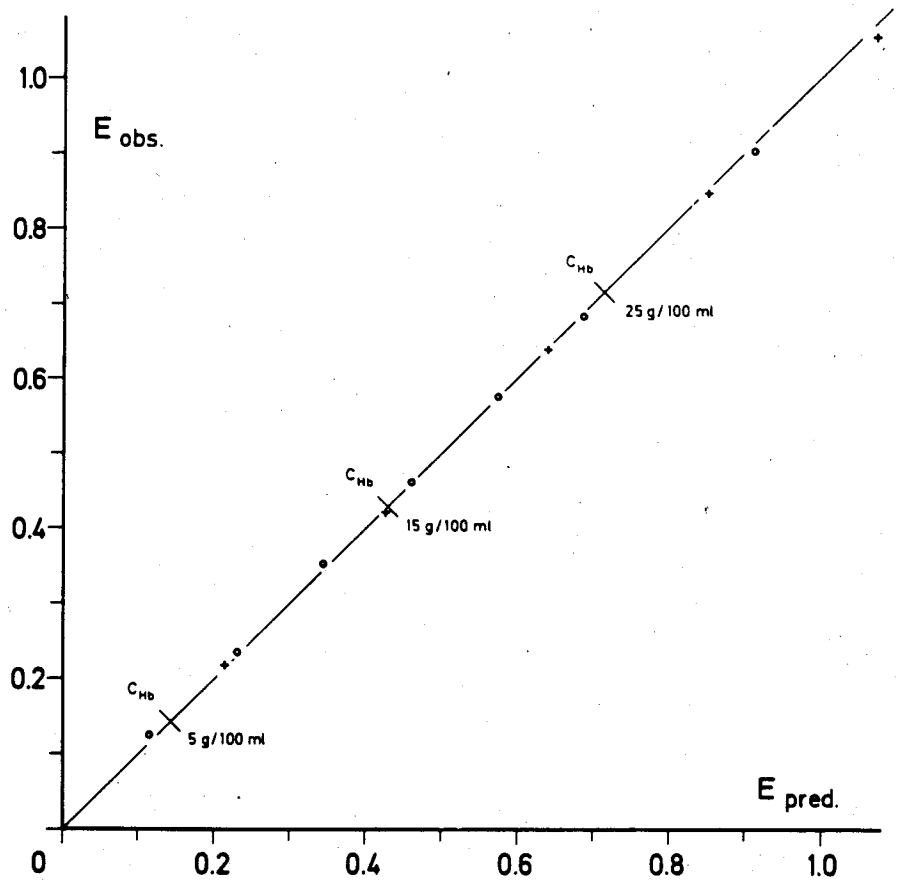

Fig. 1. Observed absorbance ($E_{obs.}$) as a function of predicted absorbance ($E_{pred.}$) for a diluted and an undiluted chlorohaemin stock solution (0.1534 mmol/l) (data of Table II, + +) and for four different stock solutions (10 mmol/l) diluted between 1/601 and 1/61 (data of Table III, ○○). The absorbance values for the theoretical c_{Hb} of 5.0, 15.0, and 25.0 g/100 ml are marked at the identity line.

differ from the calculated values (Table I) when obtained on the basis of the millimolar absorbance coefficient of 6.960, derived as shown above. They, nevertheless, show a very good linearity as can be seen in Fig. 1.

In a second series of experiments, the actual measurement of haemoglobin was simulated. 5, 10, 15, 20, 25, 30, 40, and 50 μl of a stock solution of 10 mmol chlorohaemin/l of AHD reagent were added to a constant volume of 3 ml of AHD reagent. For all dilutions tested the stock solutions were prepared from two different chlorohaemin preparations of our own and two different crystallisations of commercial crude chlorohaemin. The absorbance data for these dilutions are summarised in Table II and compared with the calculated absorbance values. As seen in the first series of experiments (Table I), the deviation of the experimental from the calculated values is also very low, and the observed data yield a straight line when plotted versus the predicted data (calculated from the amount of chlorohaemin and the absorbance coefficient) (Fig. 1).

The results obtained clearly show in both cases that in the physiological range of haemoglobin concentration — that is between 5 g/100 ml (corresponding to an absorbance of 0.143) and 25 g/100 ml (corresponding to an absorbance of 0.715) — a strong linear dependence exists between the concentration of alkaline haematin D-575 (generated from chlorohaemin or haemoglobin, respectively) and the absorbance with a deviation of $\leqslant 2\%$ of the experimental value.

Standardisation of the alkaline haematin D-575 method

The method can be standardised essentially by two different standards and procedures, i.e. using (1) cyanhaemiglobin standards commercially available, or (2) pure chlorohaemin, the latter being the standardisation procedure of choice.

(1) Standardisation by cyanhaemiglobin standards. Using cyanhaemiglobin standard solutions commercially available [concentration of haemoglobin (c_{Hb}) between

TABLE II

Experimental and calculated absorbance values of alkaline haematin D-575 dilutions obtained by adding different volumes of a stock solution of 10 mmol chlorohaemin/l (theoretical absorbance = 69.60) to 3 ml of AHD reagent (In all cases the stock solutions were prepared from two different chlorohaemin preparations and two different crystallisations of commercial crude chlorohaemin.)

Dilution ratio	Number of experiments	Absorbance ΔE		$\frac{\Delta E_{experim.}}{\Delta E_{calcul.}}$
		experimental ($\pm$ SD)	calculated	
5/3005	20	0.123 ± 0.003	0.116	1.060
10/3010	40	0.236 ± 0.004	0.231	1.022
15/3015	20	0.351 ± 0.003	0.346	1.014
20/3020	40	0.461 ± 0.005	0.461	1.000
25/3025	20	0.575 ± 0.003	0.575	1.000
30/3030	20	0.679 ± 0.004	0.689	0.985
40/3040	20	0.899 ± 0.004	0.916	0.981
50/3050	20	1.116 ± 0.004	1.141	0.978

53.3 and 212.4 mg/100 ml], different haemoglobin concentrations were prepared in a solution containing 50 g Triton X-100 in 0.2 mol/l NaOH.

The standard solutions were diluted in ratios between 1 : 1.33 and 1 : 2 by the AHD reagent until absorbance values between 0.12 and 0.69 at 575 nm (corresponding to Hb concentrations of 4.2–24.1 g/100 ml) were reached. The absorbance of these solutions was measured against a mixture, containing the AHD reagent and the HiCN reagent with the same concentrations as a reference.

Division of the haemoglobin content by the measured absorbance and multiplication by the dilution ratio of 1 : 151 yields the conversion factor, i.e. the factor for calculating the c_{Hb} (expressed as g haemoglobin per 100 ml) on the basis of the measured absorbance value. This conversion factor was obtained as

$$c_{Hb}[\text{g}/100\text{ ml}] = (34.05 \pm 0.59) \cdot \Delta E_{575} (n = 69) \tag{5}$$

(2) Standardisation using pure chlorohaemin. Since chlorohaemin preparations commercially available are not suitable as a standard because of their high content of impurities, our own preparations were used (see 'Materials and methods').

The relation between haemoglobin concentration and absorbance of alkaline haematin D-575 (formed either from haemoglobin or chlorohaemin in the AHD reagent) can be calculated as follows:

The millimolar absorbance coefficient of chlorohaemin as calculated above is

$$\epsilon_{\text{mmolar}} = 6.960 \pm 0.046\ [\text{l} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}] \tag{6}$$

1 mmol of chlorohaemin is produced by 0.25 mmol haemoglobin. Thus, the millimolar absorbance coefficient of haemoglobin, ϵ_{mmolar}(haemoglobin), in the AHD reagent is

$$\epsilon_{\text{mmolar}}(\text{haemoglobin}) = 27.84 \pm 0.18\ [\text{l} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}] \tag{7}$$

According to the Lambert-Beer law and using the dilution 1/151, the concentration of haemoglobin in mmol/l can be calculated as follows:

$$c_{Hb}[\text{mmol/l}] = \frac{151}{27.84} \cdot \Delta E_{575} = 5.424 \cdot \Delta E_{575} \tag{8}$$

or, expressed as g haemoglobin/100 ml,

$$c_{Hb}[\text{g}/100\text{ ml}] = \frac{M_r^*}{10\,000} \cdot 5.424 \cdot \Delta E_{575} \tag{9}$$

$$= \frac{64\,458}{10\,000} \cdot 5.424 \cdot \Delta E_{575} \tag{10}$$

$$= 34.96 \cdot \Delta E_{575} \tag{11}$$

* M_r, molecular weight of haemoglobin = 64458 [9].

In comparison to the conversion factor derived with cyanhaemiglobin as standard, this factor, obtained with chlorohaemin as standard, is 2.6% higher (see eq. 5).

There are two possible reasons, which have to be discussed to interpret the difference of 2.6% between both standardisation procedures.

One reason is the uncertainty of the millimolar absorbance coefficient of cyanhaemiglobin at 540 nm, and therefore, the uncertain concentrations of the standard solutions of cyanhaemiglobin.

In contrast to the value of 44.0 recommended by the ICSH based on iron analysis, a value of 43.6 on the basis of nitrogen analysis [10] or 43.5 on the basis of carbon analysis [11] have been found. Using the last value of 43.5 for calculating the conversion factor for the HiCN method, one obtains 37.19 instead of 36.77, i.e. 1.14% higher than recommended by the ICSH.

For the comparison of the two standardisation procedures described here, the consequence is as follows: because of the increase of the cyanhaemiglobin concentration of the standard solutions used, the conversion factor for alkaline haematin D-575 using cyanhaemiglobin solutions would increase from 34.05 to 34.44. Consequently, the difference between both procedures would decrease from 2.6% to only 1.5%.

Similarly, the comparison between both methods described in the preceding paper [6] would lead to another result: using the recommended conversion factor of 36.77 for the HiCN method and 34.96 for the AHD method based on chlorohaemin standardisation, the new method produces 0.5% higher values for a threefold Hb determination of 25 test persons.

If, however, a conversion factor of 37.19 is accepted, the HiCN method would give results 0.6% higher than the new AHD method. This difference might be very realistic because of the fact that all errors described for the HiCN method are positive errors and, therefore, one should expect higher values for the HiCN method compared to the AHD method.

The good compatibility of the two methods when a conversion factor of 36.77 is used indicates that the cyanhaemiglobin millimolar absorbance coefficient of 43.5 based on carbon analysis [11] might represent the true value better than the absorbance coefficient of 44.0 commonly used.

Another reason for the difference in both standardisation procedures for the new AHD method could be the fact that slight turbidity can be observed even in commercial standards [12]. This would lead to too high absorbance values even when diluted with AHD reagent. Therefore, a too low conversion factor results from standardisation of the new method using commercial HiCN standard solutions.

On account of the well-defined structure of the primary standard chlorohaemin, of the stability of this compound both in the solid and dissolved state, and of the simplicity by which standard solutions can be prepared in every laboratory, this procedure obviously is the method of choice for the standardisation of the alkaline haematin D-575 method.

Quality control

As stated in the introduction, a quality control system for clinical or laboratory

use would be desirable. In this paper such a quality control solution for haemoglobinometry is described in detail: It consists of a very stable and well-defined chlorohaemin solution of 10 mmol/l corresponding to a haemoglobin concentration of 2.5 mmol/l or, using the molecular mass of 64.458 g per millimole of haemoglobin, corresponding to a haemoglobin concentration of 16.11 g/100 ml. Thus, when the user treats a quality control solution such as blood (20 μl + 3 ml AHD reagent), under optimal conditions he should measure an absorbance value of 0.461 against the AHD reagent at 575 nm. For practical use the measured absorbance can be multiplied by a conversion factor of 35 (rounded up from 34.96), and in this case one would get a haemoglobin concentration of 16.1 g/100 ml. The advantage of such a system as a quality control is obvious.

Any error in the procedure of haemoglobin determination, e.g. errors in the measurement of the volume of the sample (20 μl) and of the reagent (3 ml) or errors in the determination of the absorbance, which can be affected by the light path of the cuvette and by the band width or the wavelength adjustment of the photometer, are excluded.

The preparation of such quality control solutions can be performed commercially as well as in every laboratory. The reproducibility of such preparations is shown in Table II. The standard deviation of 40 experiments, using four chlorohaemin preparations, 10 typical dilutions of 20/3020, and subsequent absorbance measurements amounts to only $\pm$ 1.1% of the measured value. This extremely low standard deviation includes all the following steps: chlorohaemin preparation, weighing of crystalline chlorohaemin, measurement of AHD reagent volume, preparation of the solution, measurement of 20 μl of the solution, addition of 3 ml of AHD reagent and finally determination of the absorbance at 575 nm.

Therefore, an optimal reproducibility may be reached when using a commercial quality control solution for direct use or when preparing such a solution with crystalline chlorohaemin.

Thus, the accuracy of such quality control solutions is only dependent on the purity of the crystalline chlorohaemin. A purity of more than 99%, however, should also be possible for preparations on an industrial scale *.

Hence, in combination with the haemoglobin method, the characteristics and advantages of which are described in detail in the preceding paper [6], the total procedure for determining haemoglobin by the alkaline haematin D-575 method apparently meets the recommendations of the World Health Organisation better than the conventional cyanhaemiglobin method. Therefore the question arises which of the methods should be preferred in future.

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* Note added in proof: In the meantime, chlorohaemin with a purity of > 99% can be obtained from Serva (Heidelberg, FRG).

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