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Comments

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to the paper

"Observations on the Alkaline Haematin/Detergent Complex for Measuring Haemoglobin Concentration by *O. W. van Assendelft* and *W. G. Zijlstra* (1)

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As early as March 1983 we first presented our Alkaline Haematin D-575 (AHD) method to the International Committee for Standardization in Haematology (ICSH) with a request for critical examination by recommended experts in haemoglobinometry. We adopted approach because we considered the AHD method to be a possible candidate for an ICSH selected or even recommended method; it seemed to meet most of the ICSH requirements for an analytical procedure. However, all expectations that the ICSH would thoroughly compare our method with the reference cyanhaemiglobin (HiCN) method according to the strong criteria of the ICSH (e. g. accuracy, precision, and possibility of calibration) have been disappointed by the behaviour of the ICSH and by the preceding publication by *van Assendelft & Zijlstra* (chairman and member of the ICSH, respectively) mentioned above.

It is clear that our method was not fairly examined or rigorously studied by independent authorities in the same way as the HiCN method. Instead of presenting comparable and comprehensive data for both methods (AHD and HiCN) in all respects (spectra, stability, conversion rates, method-related errors, advantages and disadvantages), only some aspects were examined arbitrarily (reaction of normal and fetal blood), and advantages of our method (low turbidity, rapid conversion of HbCO) were considered to be marginal. In addition, other most important aspects, such as the possibility of direct standardization (with crystalline haemin) and the performance of a reliable quality control (with a solution of 10 mmol/l haemin in AHD reagent) were not even mentioned in their paper (1). Therefore, we feel obliged to give the following clear response to this paper.

Comments concerning the correct use of ICSH criteria

The ICSH has established criteria for defining a method as definitive, reference or selected with decreasing requirements. Obviously, *van Assendelft & Zijlstra* did not use these criteria appropriately, because the requirements demanded by them for the AHD method as a possible candidate for a selected method are definitely higher than those demanded for the existing reference method (HiCN). This may be demonstrated by two characteristic examples:

Example 1

From the data of a survey on the reliability of HiCN standard solutions (calibrators) commercially available on the US market (l. c. (2), table 2), it is evident that in some cases the measured contents may differ up to 6.6%, a deviation which was interpreted by the author (*van Assendelft*) as: "The inaccuracy of the assigned value is usually not relevant clinically."

On the other hand, the same author, when testing three different detergents in the AHD method for determination of haemoglobin in 11 normal blood samples, and obtaining the following results in comparison with the HiCN method (% deviation relative to normal Hb of 150 g/l):

Triton X-100:	+1.0 %,
Siponic 218:	−0.4 %,
Sterox SE:	−0.03%,

concluded in the summary: "... that different non-ionic detergents in the reagent result in identical values for the haemoglobin concentration being measured could not be verified." This statement was made in spite of the fact that in the case of our method the deviation was lower by at least one order of magnitude than in the case of the reference method!

Example 2

Van Assendelft & Zijlstra note that the absorbance spectrum of the haematin detergent complex (l.c. (3), fig. 2) is influenced by the type of the starting material (haemin, whole blood, HiCN standard solutions), but no comparable spectra of the corresponding HiCN complexes over the spectral range of 500−750 nm derived from the same starting material are presented. However, careful inspection of the cited 521 determinations (l.c. (4), tab. 3) of the millimolar absorptivity value ε^{540}_{HiCN}, also clearly shows an influence of the starting material: a difference of 1.4% between human whole blood (n = 5, 3 papers) and purified human haemoglobin (n = 8, 6 papers), and a difference of 1.4% between fetal whole blood and the generally accepted value of 11.0!

Comments on the kind of examination of our method

Three characteristic examples may demonstrate that the kind of examination of our method has to be designated as "not fair":

Example 1: "Alinearity"

Van Assendelft & Zijlstra use data from our publication (l.c. (5), tab. 2) to demonstrate alinearity between the absorbance of the haematin/detergent complex at the maximum wavelength of 575 nm (A_{575}) and the haemin concentration c (mmol/l), (see tab. 1 and fig. 2 in l.c. (1)). Treatment of the data (n = 8) for a best linear fit yields an equation for a straight line, which is highly significant (r = 0.99997):

$$A_{575} = 6.73 \times c + 0.0141$$

i.e., linearity is certainly given, however, with an intercept of 0.014, slightly different from zero.

In figure 2 of the paper of *van Assendelft & Zijlstra* (l.c. (1)), the measured absorbances are wrongly plotted in two ways:

1) Abscissa: On the abscissa, the concentration scale is divided into 10 equidistant spaces corresponding to the range of dilution from 5/3005 to 50/3050. However, e.g. the highest concentration is not 10-fold of the lowest as marked in the plot, but only 9.85-fold and therefore all concentration marks on the scale shift to the left, increasingly so as the concentration increases.

2) Ordinate: Some of the measured absorbance values, i.e. 0.123, 0.236, and 0.351 are obviously plotted wrongly, and the line drawn through these measured absorbances therefore is shifted generally to higher ordinate values. This error can be seen especially at the intercept of 0.045, a value more than threefold higher than the true value of 0.014 (see above).

Example 2: AHD spectrum from whole blood

In figure 1 of the paper by *van Assendelft & Zijlstra* (l.c. (1)) the time course of the spectrum of the haematin/detergent complex produced from human whole blood is shown over a period of 48 h. Instead of Triton X-100, which is the preferred detergent recommended by us for the AHD reagent, they used Sterox SE in this examination. What was the rationale for not using Triton X-100?

In addition, no comparable spectrum for the HiCN reagent and whole blood in the same spectral range and over the same time is presented.

Example 3: "Correct values only after (re)calibration"

One of the authors, *W. G. Zijlstra*, who recommends the new method HiN_3 (HemoCue®), together with other authors in a recent paper (l.c. (6), p. 408) describes a mean systematic deviation of −3.4% in comparison with the HiCN method. In spite of this result, *Zijlstra* states that the HemoCue® system "... measures accurately ..." and "... gives results identical with reference HiCN method only after correct (re)calibration." Is there no difference between a "(re)calibration", using a "red control cuvette" for the empirical calibration of the new method HiN_3, and the crystalline standard of the AHD method, commented on by *van Assendelft & Zijlstra* (1) as "... although it has some merit."?

Comments on calibration and quality control

We agree that the choice of method, HiCN or AHD, does not matter in the daily clinical routine haemoglobinometry. However, we definitely do consider that the examination of the AHD method should be performed properly, since it represents an opportunity to introduce a method

1) which can be standardized by a chemically pure substance (haemin) of exactly defined composition, of which weighed amounts can be used to adjust the concentration accurately (2), and

2) which can be reliably controlled in all steps from taking blood, sampling, dilution and photometric measurement, using a defined quality control procedure (using a solution of 10 mmol/l haemin in AHD reagent).

It seems paradoxical, when — in the discussion about a systematic bias of 2.6% between the two methods — *van Assendelft & Zijlstra* (1) argue that there is no guarantee that our haemoglobin determinations with the HiCN method were correct, when we used commercial reagents and standards in the past (2). What could describe better the critical situation concerning standards and reference solutions in the HiCN method?

At least, we would have expected that the ICSH, which always emphasizes that haemoglobinometry must be dynamic and open to new developments and improvements, would have tested the standardization with both crystalline haemin and HiCN standard solutions. All doubts about the true contents of the HiCN reference solutions could have been excluded by choice of an optimum solution prepared and checked by ICSH reference laboratories. By conversion of known HiCN solution into the alkaline haematin/detergent complex by means of an AHD reagent of an appropriate concentration, the millimolar absorptivity value of HiCN (ε^{540}_{HiCN}) could have been controlled on a haemin basis and indirectly also on an iron basis.

We certainly know that the AHD method is not yet perfect and that many questions, such as the exact nature of the detergent complex or the mechanism of its formation, are still open. But the prospect of a method, which can be standardized by a well-defined chemical substance simply by weighing, justifies its further development and is a good rationale for testing this method without any prejudice.

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