J. Clin. Chem. Clin. Biochem.
Vol. 27, 1989, pp. 181–184

Editorial

The Determination of Haemoglobin in Whole Blood

Diverging Developments in Clinical Chemical Analysis: Critique and Commentary

In looking through recent scientific publications on quantitative analytical methods for clinical chemical parameters (quantities) the critical reader will fail to find a consensus among the authors about the goals of their work.

Although many different questions are being addressed, two major objectives can be identified – objectives that often run counter to each other.

For more than 20 years one objective has been to obtain reliable analytical results, despite the quite variable biological matrix. That is, the regularly monitored imprecision and inaccuracy and the related analytical specificity should meet the medical requirements.

The other frequent objective is to make analyses as simple, quick and inexpensive as possible. In this context, it is considered an advantage if the analyses can be performed by people with only brief on-the-job training.

This issue of the Journal of Clinical Chemistry and Clinical Biochemistry includes two articles (1, 2) on the determination of haemoglobin in whole blood. The second article (2) contains observations on the first (1). The two papers are followed by a commentary (3). The editors have decided to publish these controversial papers (1, 2) and the subsequent commentary (3) to sharpen the reader's awareness of extremely disparate developments.

This editorial serves the same purpose; it includes a brief discussion of the following:

1. the development of the standardized method for determining haemoglobin in whole blood;
2. the role of reference methods in the New Concept for quality control in clinical laboratory investigations;
3. the haemiglobin cyanide (HiCN; cyanmethaemoglobin) method for routine investigations;
4. the "alkaline haematin D-575" method.

Since the end of the last century the determination of the haemoglobin concentration of whole blood has been one of the most frequently conducted and widely used investigations in the field of medicine. The development of methods for analysing this quantity began well before the turn of the century (4). For these reasons, and because of the great variety of analytical principles employed over the years, haemoglobin serves well to illustrate the progress made in analytical techniques and in the critical evaluation of methods in clinical chemistry (5, 6).

1. The development of the standardized method

For many years, because of the wide variety of analytical principles employed in determining haemoglobin in whole blood and the different methods (including calibration) used, the results of such determinations were unreliable and therefore not really comparable. This was an unacceptable situation.

At the beginning of the 1950s an Ad Hoc Panel for the Establishment of a Hemoglobin Standard was set up by the Division of Medical Sciences of the National Academy of Sciences, USA, to evaluate a great many of

the methods then in use; this panel came to the conclusion that conversion of haemoglobin to haemiglobin cyanide was the best analytical principle available (7). The reasons are as follows:

1. the blood is diluted with only a single reagent;
2. all forms of haemoglobin found in circulating blood are measured except sulphaemoglobin (SHb). SHb is normally present in a concentration of less than 0.1 percent (except in intoxication);
3. the absorbance (molar lineic absorbance) of the haemiglobin cyanide formed can be measured both with filter photometers and with high resolution spectrophotometers;
4. the calibrators can be prepared from crystalline haemoglobin or from washed erythrocytes; the purity of the calibrators can be checked, and the concentration can be determined with suitable independent analytical procedures.

Since 1960, as evidenced in the papers by *E. J. van Kampen* and *W. G. Zijlstra* (8, 9), the protocol for the method, including specifications for the calibrators with the associated criteria for purity and the spectral properties, has been described in detail and evaluated; this has included all measures necessary to ensure reliability.

After extensive comparative investigations, the International Committee for Standardization in Hematology (ICSH) recommended this method for determination of haemoglobin in human blood. Since then the method has been subjected to repeated critical evaluations, which have led to the 1977 and 1987 revisions of the Standard Method (10).

The analytical principle on which the standardization is based proved to be so well suited for haemoglobin determination that it then served as the basis for the development of a reference method.

2. The role of reference methods

Comparability of analytical results from the same laboratory or from different laboratories is assured if random errors (imprecision) and systematic errors (inaccuracy) are monitored on an ongoing basis and remain within the limits specified with reference to the medical requirements. The medical requirements have been met if the percentage of incorrect (false positive and false negative) classifications in the medical assessment step is kept within the limits specified. The inaccuracy of analytical systems is monitored by comparing the analytical results with reference method values (where available) or with other values that are equally good estimates of the "true value".

This principle is also the basis for the maximum allowable random and systematic errors specified in the new Guidelines of the Bundesärztekammer (Federal Medical Association) (11) that recently went into effect in connection with a revised version of the Eichgesetz (calibration act, verification act) (12) and the related Eichordnung (weights and measures regulations) (13).

The development of a reference method for a given quantity involves the following steps:

1. definition of the quantity (system, analyte, kind of quantity) to be determined;
2. specification of the demands made on the reliability criteria for the method;
3. selection of the analytical principle for the reference method;
4. development and validation of the candidate reference method;
5. assessment of the transferability of the reference method (i.e. implementation of the method in other laboratories);
6. approval as a reference method by international or national bodies;
7. recommendation and use of the reference method;
8. evaluation and revision of the reference method at regular intervals.

A routine method and a reference method can be based on the same analytical principle. However, if an analytical principle serves as the basis for development of a reference method, then a large number of

additional measures must be taken to ensure that the reference method is reliable. Consequently, reference methods can be developed and used only in a small number of laboratories with especially highly qualified academic and technical personnel.

Reference methods are an essential prerequisite for national and international reference systems to serve as the basis for valid measurements in the field of medicine.

For the determination of haemoglobin in whole blood, a reference method based on the haemiglobin cyanide method has been developed and approved by the ICSH (14).

Since then the World Health Organization (WHO) has accepted and promoted the haemiglobin cyanide method as both a reference method and a routine method. To this end they have also made ampules of haemiglobin cyanide available for standardization (14).

3. The haemiglobin cyanide method in the routine laboratory

Conversion of haemoglobin to haemiglobin cyanide is also the principle on which a reliable routine procedure is based that yields analytical results close enough to the "true value" to satisfy the medical requirements. For analytical results that do not fit the clinical picture, laboratories under highly qualified directorship must have available a selected analytical method for studying patient specimens which enables them to determine the causes of the deviations. Over the past 20 years the haemiglobin cyanide method has proved to be particularly well suited for this purpose. Before implementation, any new routine method must be evaluated with the same thoroughness that has been accorded to the haemiglobin cyanide method.

Up to now the known reliability of the haemiglobin cyanide routine procedure has outweighed the time and expense involved in taking the special precautions required with this procedure.

4. The new "alkaline hematin D-575" method

In this method (15), 20 µl of blood is mixed with 3 ml of an "AHD reagent" solution; this "AHD solution" contains 25 g of the detergent (the "D" in the method name) Triton X-100 in 1 l of 0.1 mol/l NaOH. An olive-green substance results that has an absorbance peak at 575 nm. There are still many open questions about the chemical nature and characterization of this substance. To calibrate the haemoglobin determination (16) a chlorohaemin is used, for which information on purity and results of elementary analyses have not yet been published.

So far the evaluation of the method has consisted mainly of determination of the imprecision, and a comparison of pairs of analytical results obtained with the haemiglobin cyanide method and the "alkaline hematin D-575" method from samples of the same specimen and evaluated statistically.

Conclusions

The haemiglobin cyanide method was evaluated very carefully during its development in the 1960s (17, 18). The results of the evaluations have since been confirmed by independent investigators (8, 9). The calibrator for this determination has been characterized exactly and its (milli)molar lineic absorbance has been determined and confirmed on the basis of analyses with independent analytical principles (e. g. iron determination and elementary analysis) [n = 16; (9), p. 204].

In contrast, the "alkaline haematin D-575" method was initially evaluated only by the investigators who first described it (15, 16). Since then a test of the method by *van Assendelft & Zijlstra* (2) has raised a good many questions. The characterization of the pigment on which the measurement signal is based and the necessary information on the purity of the calibrator and on independent analyses of the calibrator are not yet available. In other words, the method has not yet been adequately validated. A statistical comparison of analytical results obtained with the method being assessed and results obtained with a validated method from samples of the same specimens is not a substitute for a careful validation of an analytical method.

In view of our current state of knowledge, the "alkaline haematin D-575" method cannot be regarded as equivalent to the haemiglobin cyanide method, and it clearly cannot replace the latter method at present.

Analytical principles that have proven reliable should not be replaced by new analytical principles that appear to have advantages, until the validity of the new principles has been established in independent laboratories. A statistical comparison of methods can supplement such a validation process, but it cannot take its place. The validation process must demonstrate that the reliability criteria for the new method satisfy the medical requirements.

It is the hope of the editors that the detailed papers appearing in this issue will lead to a methodological discussion on the determination of haemoglobin in whole blood.

Dankwart Stamm, Munich

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Prof. Dr. Dr. D. Stamm
Max-Planck-Institut für Psychiatrie
Abteilung für Klinische Chemie
Kraepelinstraße 10
D-8000 München 40