International Committee for Standardization in Haematology


PREAMBLE

Scientific symposia on haemoglobinometry were held at the 9th Congress of the European Society of Haematology, Lisbon, 1963 (Erythrocytometric methods and their standardization. Bibliotheca Haematologica (Basel), 1964, 18), and the 10th Congress of the International Society of Haematology, Stockholm, 1964 (Standardization, documentation, and normal values in haematology. Bibliotheca Haematologica (Basel), 1965, 21).

An expert panel on haemoglobinometry was set up in Stockholm on 2 September 1964. On the basis of discussions by this panel the International Committee for Standardization in Haematology (ICSH) made recommendations for a reference method for haemoglobinometry and for the manufacture and distribution of an international reference preparation. These recommendations were endorsed by the General Assembly of ICSH in Sydney on 23 August 1966.


On the basis of continued experimental studies the reference method and the specifications for the international reference preparation have been modified.


Haemoglobin is a chromoproteid. On the basis of the chemical structures of two α and two β chains and of four haem groups it is calculated to have a relative molecular mass of 64 458 (anhydrous) (Braunitzer et al., 1961, 1964; Hill et al., 1962). The mass fraction of haemoglobin iron is then 0·003 47.

At its 9th Assembly in Kyoto, Japan, in September 1976 ICSH recommended that at the present time the haemoglobin content of blood should be expressed as mass concentration in grammes per litre. In conformity with the joint recommendation for use of the international system of units (SI) in clinical laboratory measurements, as agreed by the International Committee for Standardization in Haematology (ICSH), the International Federation of Clinical Chemistry (IFCC), and the World Association of Societies of Pathology (WASP) (1972, 1973), it is, however, permitted to use substance concentration (mmol per l). In that case the elementary entity (monomer or tetramer) should be specified—that is, by use of the notation (Fe) or (4 Fe). For measurement of the reference preparation the expert panel recommends relating molar absorp-
tion coefficient and relative molecular mass to one haem group and one quarter of the total globin moiety.

**PRINCIPLE**

Photometric determination of haemoglobin cyanide is recommended as the reference method. If any other method is used (for example, photometric determination of oxyhaemoglobin, iron determination, gas analytic methods) it should be adjusted to obtain comparability with the haemoglobin cyanide method. The determination of haemoglobin as haemichloride (acid haematin) is not recommended because of the unreliability of this method.

**REAGENT**

The haemoglobin derivatives existing in blood, with the exception of verdoglobin (sulphaemoglobin), are converted into haemoglobin cyanide by the use of an appropriate reagent. This must be of such a quality that after dilution of the blood there is no turbidity. To assure complete conversion the photometric determination must be delayed until the reaction is completed.

**EXTINCTION MEASUREMENT**

When a spectrophotometer is used the blood should be diluted suitably (1:251) with the reagent and measured at 540 nm (or with a mercury lamp at the mercury line 546 nm) against an appropriate blank.

When a photoelectric colorimeter (filter photometer) is used the blood should be diluted suitably (1:251) with the reagent and measured through a narrow band yellow-green filter with maximal transmission near 540 nm against an appropriate blank.

In each case the instrument must be calibrated and the linearity verified by means of a sterile or membrane-filtered (at pore size 0.2-0.25 μm) haemoglobin cyanide calibration standard using a procedure such as that described, for example, by Eilers and Crocker (1972). Even minor changes in the set up of the method may cause significant deviations in calibration.

When a photoelectric colorimeter is used the condition of the filter should also be checked at intervals to ensure that no defect has developed.

**CALIBRATION STANDARD**

The haemoglobin cyanide calibration standard should be an aqueous solution of haemoglobin cyanide with a concentration in the range of 550-850 mg/l. It is strongly recommended that it be dispensed as a sterile solution in individual doses in sealed ampoules of amber glass. The spectrophotometric characteristics must conform to the following specifications.

**Content**

The HiCN (equivalent haemoglobin) content is calculated from

\[ c(\text{mg/l}) = \frac{A_{540}^{\text{HiCN}} \times 16\,114.5}{11.0 \times 1} = 1465 \times A_{540}^{\text{HiCN}} \]

where \( A_{540}^{\text{HiCN}} \) is absorbance of the solution at \( \lambda = 540 \) nm,

\[ 11.145 \text{ relative molecular mass of haemoglobin} \left( \text{derived from} \frac{64458}{4} \right), \]

\[ 11.0 = e_{540}^{\text{HiCN}} \text{ (millimolar absorption coefficient)}, \]

1 = light-path length in cm, to be known with an accuracy to three decimal places.

1 Alternative terms are cyanmethaemoglobin and cyanferrihaemoglobin.

2 A suitable reagent (recommended by van Kampen and Zijlstra (1961)) is prepared as follows. Dissolve 200 mg of \( \text{K}_2\text{Fe(CN)}_6 \), 50 mg of KCN, 140 mg of \( \text{KH}_2\text{PO}_4 \) (analytic grade chemicals), and an appropriate amount of non-ionic detergent in water and dilute to 1 litre. The pH should be 7.0-7.4 (pH meter). If stored at room temperature in a brown borosilicate glass bottle the solution keeps for several months. It should be controlled regularly. It must not be allowed to freeze. Examples of non-ionic detergents are Nonic 218 (Pennsalt Chemicals) 1 ml/l, Nonidet P40 (Shell International) 1 ml/l, Quolac Nic 218 (Unibasic) 1 ml/l, Sterox SE concentrated (Hartman-Leddon) 0.5 ml/l, Triton X-100 (Rohm and Haas) 1 ml/l. They are available in general from laboratory chemical suppliers.

This reagent has a conversion time of 3 min in contrast to the original or the modified Drabkin's reagent with a conversion time of 10 min or more. Drabkin's reagent consists of \( \text{NaHCO}_3, 1 \text{g, K}_2\text{Fe(CN)}_6, 200 \text{mg, KCN 50 mg}, \) and distilled water to 1 litre. It has a pH of 8.6. The modified reagent without the \( \text{NaHCO}_3 \) has a pH of 9.6. Because of the longer conversion time as well as the greater danger of turbidity, these Drabkin's reagents are not recommended.

3 More precisely 546.1.

4 Solutions of lower concentration are not reliable.

5 See Zijlstra and van Kampen (1960) and van Assendelft and Zijlstra (1975).
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Using an appropriate blank $A_{540}^{HICN}$ is measured on a spectrophotometer, the wavelength scale of which has been calibrated with the aid of the Hg (or H) emission spectrum and absorption checks have been performed using calibrated glass filters or other means which have been tested by independent standardising laboratories. Its slit width is so chosen that the half intensity band-width is less than 1 nm. The cuvettes in which the calibration standard is measured are plan-parallel with an inner wall-to-wall distance of 1,000 cm, tolerance 0-5% (0-995-1-005). The measurements are carried out at 20-25°C.

Purity

The purity is controlled by (1) Judging the shape of an absorption spectrum between $\lambda = 450$ and 750 nm, light-path length 1-000 cm. (2) Determining the quotient $A_{540}^{HICN}/A_{504}^{HICN}$. The value of this quotient should lie between 1-59 and 1-63. (3) Measuring in near infrared to check turbidity (between $\lambda = 710$ and 800 nm—for example, at $\lambda = 750$ nm). The absorbance should be less than 0-002 per cm light-path length.

Stability

The label of the container must indicate an expiry date after which the material must not be used (see below under ‘Stability’ of haemiglobincyanide reference preparation).

Determination of Haemoglobin Concentration

If measured on a photoelectric colorimeter (filter photometer) the haemoglobin concentration is read from a previously constructed calibration graph or, where applicable, directly from the scale.

If measured on a spectrophotometer the haemoglobin concentration is calculated using the equation

$$c(g/l) = \frac{A_{540}^{HICN} \times 16 114-5 \times F}{11-0 \times 1}$$

where $A_{540}^{HICN} =$ absorbance of the solution at $\lambda = 540$ nm,

16 114-5 = relative molecular mass of haemoglobin (derived from $64\,458/4$),

$$F = \text{dilution factor used (for example, } 1:251),$$

$$11-0 = \epsilon_{540}^{HICN} \text{ (millimolar absorption coefficient),}$$

$1 = \text{light-path length in cm.}$

For a dilution (F) of 1:251

$$c(g/l) = 367-7 \times A_{540}^{HICN}.$$


Manufacture

The international haemiglobincyanide reference preparation is manufactured at three-year intervals on behalf of ICSH by the Rijks Instituut voor de Volksgezondheid, Bilthoven, the Netherlands. Details of the method are given by Holtz (1965). It is made from washed human red cells, haemolysed by toluene, and centrifuged free from debris. The haemoglobin is converted to haemiglobincyanide. The final solution is equivalent to a haemoglobin content of approximately 600 mg/l. It is dispensed as a sterile solution (membrane filtration) in sealed 10-ml ampoules of amber glass. A batch of this reference preparation has been designated by WHO (1968) as International Haemiglobincyanide Reference Preparation.

Evaluation and Control

Each batch is tested in laboratories, nominated by

*It was agreed that whereas the international reference preparation would be aqueous national boards might provide glycerinated solutions if preferred.
*At present these laboratories are Atlanta (USA), Hematology Division, Center for Disease Control (O. W. van Assendelft); Cleveland (USA), Standards Laboratory of the College of American Pathologists, Cleveland Clinic (C. E. Willis); Freiburg i.Br. (Germany), Institut für Standardisierung und Dokumentation im Medizinischen Laboratorium, Medizinische Universitätsklinik (A. von Klein-Wisenberg); Groningen (the Netherlands), Diaconessenhuis (E. J. van Kampen), and Laboratorium voor Vegetatieve Fysiologie, Rijks Universiteit (W. G. Zijlstra); Kumamoto (Japan), Department of Laboratory Medicine, Kumamoto University Medical School Hospital (T. Matsubara); London (England), King’s College Hospital Medical School (J. M. White); Rome (Italy), Istituto Superiore di Sanità (L. Tentori); Washington (USA), Bio-organic Standards Section, Analytical Chemistry Division, National Bureau of Standards (R. Schaffer).
the ICSH Board, in accordance with the principles set out below. The results are analysed by a consultant who advises the ICSH Secretariat on the control of the preparation.

Content

The HiCN (equivalent haemoglobin) content is calculated from the results of at least five laboratories, using the equation

\[ c(\text{mg/l}) = \frac{A_{540} \times 16.114.5}{11.0 \times 1} = 1465 \times A_{540}^{540}, \]

where \( A_{540} \) = absorbance of the solution at \( \lambda = 540 \) nm,

\[ 16114.5 = \text{relative molecular mass of haemoglobin} \quad \left( \text{derived from} \quad \frac{64458}{4} \right), \]

\[ 11.0 = \varepsilon_{540}^{540} \text{ (millimolar absorption coefficient)} , \]

\[ 1 = \text{light-path length in cm, to be known with an accuracy to three decimal places.} \]

Using an appropriate blank, \( A_{540}^{540} \) is measured on a spectrophotometer the wavelength scale of which has been calibrated with the aid of the \( \text{Hg} \) (or \( \text{H} \)) emission spectrum and absorption checks have been performed using calibrated glass filters or other means which have been tested by independent standardising laboratories. Its slit width is so chosen that the half intensity band-width is less than 1 nm. The cuvettes in which the solution is measured are plan-parallel with an inner wall-to-wall distance of 1-000 cm, tolerance 0-5% (0-995-1-005). The measurements are carried out at 20-25 °C.

The results are correlated at the Rijks Instituut voor de Volksgezondheid. The statistical mean of the results of the laboratories is recorded after erratic results have been discarded in accordance with statistical practice. Experience with this procedure has shown that the confidence limits of the final results are within ± 1%.

Purity

The purity is controlled by (1) Judging the shape of an absorption spectrum between \( \lambda = 450 \) and 750 nm, light-path length 1-000 cm. (2) Determining the quotient \( A_{540}^{540} / A_{540}^{540} \). The value of this quotient should lie between 1·59 and 1·63. (3) Measuring in near infrared to check turbidity (between \( \lambda = 710 \) and 800 nm—for example, at \( \lambda = 750 \) nm). The absorbance should be less than 0·002 per cm light-path length, using an appropriate blank.

Stability

The International Reference Preparation is kept at 4°C. It has a stability of at least six years. This stability is checked by the testing laboratories repeating the primary standardisation several times per year. The manufacturing laboratory repeats absorbance measurements at more frequent, regular intervals.

Sterility

In conformity with current practice of sterility control the contents of the ampoules to be tested are inoculated in aerobic and anaerobic media and incubated at 22 and 37 °C.

Labelling

The International Reference Preparation is labelled with the batch number, the value of its haemoglobin content (mean and standard error, see above under 'content'), and an expiry date which is considered well within safe limits (see under 'Stability'). The producer notifies all consumers if continuing control of stability indicates that a particular batch is no longer acceptable.

Distribution

The International Reference Preparation is made available for reference use only to national standards committees for haematological methods or to official government-nominated holders. When there is no committee or official holder it is distributed to an individual appointed by ICSH.

The national holder must ensure that an opportunity is given to manufacturers and distributors to use the International Haemoglobin cyanide Reference Preparation as a reference material if so desired, in conformity with national requirements.

Information about national committees, official

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10 See Zijlstra and van Kampen (1960) and van Assendelft and Zijlstra (1975).
12 See van Kampen et al. (1965).
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holders, and other contact persons may be obtained from the ICSH Secretariat, c/o Dr. S. M. Lewis, Royal Postgraduate Medical School, Ducane Road, London W12 OHS, United Kingdom.

References


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