International Committee for Standardization in Haematology of the European Society of Haematology

Recommendations and requirements for haemoglobinometry in human blood

Scientific symposia on haemoglobinometry were held at the ninth congress of the European Society of Haematology at Lisbon in 1963 (see 'Erythrocytometric methods and their standardization' Bibl. haemat., 1964, Fasc. 18) and at the tenth congress of the International Society of Haematology at Stockholm in 1964 ('Standardization, documentation and normal values in haematology', Bibl. haemat., 1965, fasc. 21).

A technical committee on haemoglobinometry met in Stockholm on 2 September 1964 to discuss problems associated with the production of a standard solution. On the basis of this discussion and the conclusions reached at the symposia the following recommendations have been made by the International Committee for Standardization in Haematology (I.C.S.H.).

**DEFINITION**

Haemoglobin is a chromoprotein. On the basis of the chemical structures of its α and β chains and of haem it is tentatively assumed to have a molecular weight of 64458 (Braunitzer, Gehring-Mueller, Hilschmann, Hilse, Hobom, Rudloff, and Wittmann-Liebold, 1961; Hill and Konigsberg, 1962; Braunitzer, 1964). Assuming the presence of 4 atoms of iron in the molecule the iron content will be 0.347%, provided that H₂O is absent. Data concerning the haemoglobin content of blood should be expressed in grams per 100 ml.

**METHOD**

Photometric determination of haemoglobin cyanide (cyanmethaemoglobin) is recommended as the standard method. If any other method is used (e.g., 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 haeminchloride (acid haematin) is not recommended because of the inaccuracy 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 conversion of haemoglobin is complete.

**EXTINCTION MEASUREMENT**

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

When a photoelectric colorimeter (filter photometer) is used the blood should be suitably diluted, e.g., 1:251, with the reagent and measured through a yellow-green filter with maximal transmission near 540 nm., e.g., Ilford 625.

When a visual-reading haemometer is used the blood should be diluted with the reagent and the measurement carried out in accordance with the instructions of the manufacturer.

**STANDARD**

In each case the instrument must be calibrated by means of a haemoglobin cyanide standard solution. This solution should be manufactured in a way similar to that used for the preparation of the I.C.S.H. reference standard (see below) and it should be related to that international standard. A check of the calibration of the instrument by

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1A suitable reagent with a short conversion time (recommended by van Kampen, E. J., and Zijlstra, W. G. (1961), Clin. Chim. Acta, 6, 338) contains the following constituents dissolved in 1 litre of water: (1) potassium ferricyanide 200 mg., (2) potassium cyanide 50 mg., (3) potassium dihydrogen phosphate 140 mg. (4) A suitable quantity of a colourless surface-active agent (0.5 ml. of Sterox S.E. (Hartman-Leddon Co. of Philadelphia) or 1 ml. of Nonidet P40 (Shell Chemical Co. of London) have been successfully used). The pH should be 7.0 - 7.4 (checked by pH meter); if stored at room temperature in a black polythene bottle, the solution keeps for several months, but should be checked regularly. It must not be allowed to freeze.
means of the standard solution should be performed at regular intervals. Even minor changes in the set up of the method may cause significant deviations in calibration. When a photo-electric colorimeter is used, the condition of the filter should also be checked at intervals to ensure that no defect has developed in its transmission efficiency.

I.C.S.H. HAEMOGLOBINCYANIDE REFERENCE STANDARD

The reference standard is prepared on behalf of the Committee by the Rijks Instituut voor Volksgezondheid, Utrecht, Netherlands. Details of the method are given by A. H. Holtz (Bibl. haemat., 1965, Fasc. 21). The standard consists of human washed red cells, haemolysed by toluene, and centrifuged free from debris. The haemolysate is converted to haemiglobincyanide. It is equivalent to a haemoglobin content of approximately 60 mg. per 100 ml. It must be used as a sterile solution and is dispensed in 10 ml. ampoules of amber glass.

Each batch is tested by five laboratories nominated by the Committee in accordance with the principles which are detailed in the appendix.

The standard will be labelled with the batch number, with the value of its haemoglobin content (mean and standard deviation) and with an expiry date. Users will be notified if continuing control of stability indicates that a particular batch is no longer acceptable prior to the expiry date.

The standard will be made available, for reference use only, to national standards committees for haematological methods or to official government-nominated holders. Where no committee or official holder exists it might be distributed to an individual appointed by I.C.S.H. The national holder must ensure that manufacturers and distributors are given the opportunity to have their product checked against the international standard in conformity with national regulations.

In Britain the use of the standard is being controlled by the British Committee for Standards in Haematology in collaboration with the British Standards Institution. Standard solutions which are prepared in accordance with the principles laid down and which conform to the I.C.S.H. specifications may be labelled accordingly by the manufacturer. Supplies of standard solution, for use in routine haemoglobinometry, are now available commercially.

A British standard for cyanmethaemoglobin (haemiglobincyanide) solution for photometric haemoglobinometry (B.S.S.) conforming to the I.C.S.H. specifications is being prepared. Enquiries concerning the reference standard should be addressed to Professor I.D.P. Wootton, Postgraduate Medical School, Ducane Road, London, W.12. or to I.C.S.H. Secretariat, Sterrenbos 1, Utrecht, Netherlands.

APPENDIX

EVALUATION AND CONTROL OF I.C.S.H. HAEMOGLOBINCYANIDE REFERENCE STANDARD

1. CONTENT

The haemoglobin content is calculated from

\[ C(\text{mg./100 mL}) = \frac{D_{540}^{\text{HiCN}} \times 64458}{44.0 \times d \times 10} \]

where:

- \( D_{540}^{\text{HiCN}} \) = optical density of the solution at \( \lambda = 540 \) nm,
- \( 6458 \) = molecular weight of haemoglobin,
- \( 44.0 = E_{540}^{\text{HiCN}} \) (millimolar extinction coefficient),
- \( d \) = layer thickness in cm, to be known with an accuracy to three decimal places,
- \( 10 \) = conversion factor from 1 litre to 100 ml.

\( D_{540}^{\text{HiCN}} \) is measured on a spectrophotometer of which the wavelength scale has been calibrated with the aid of the Hg (or H) emission spectrum and absorption checks have been performed. Its slit width is so chosen that the half intensity band width is less than 1 nm.

The cuvettes in which the standard is measured are panparallel with an inner wall-to-wall distance of 1-000 cm., tolerance 0.5% (0.995 to 1.005 cm.).

Values for \( D_{540}^{\text{HiCN}} \) are corrected to 23°C. Results are correlated at the Rijks Institut voor Volksgezondheid. The statistical mean of the results from the five laboratories is recorded; erratic results are discarded in accordance with statistical practice.

2. PURITY

The purity is controlled by:

2.1 Judging the shape of a \( \lambda D \) curve between \( \lambda = 700 \) nm. and \( \lambda = 450 \) nm, layer thickness 1,000 cm.

2.2 Determination of the quotient

\[ \frac{D_{540}^{\text{HiCN}}}{D_{504}^{\text{HiCN}}} \]

The value of this quotient should lie between 1.58 and 1.62.

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Footnotes:

1. It was agreed that whereas the international reference solution would be aseptic, national boards might provide glycerinated solution if preferred.

2. At present these laboratories are:
   - Cleveland (U.S.A.), Reference Laboratory, College of American Pathologists, Cleveland Clinic, Cleveland, Ohio (J. W. King).
   - Freiburg i Br. (Germany), Medizinische Universitatsklinik (A von Klein-Wisnenberg).
   - Groningen (Netherlands), Fysiologisch Laboratorium Rijks Universiteit (W. G. Zijlstra) and Diaconessenhuis (E. J. van Kampen).
   - London (United Kingdom), Postgraduate Medical School (I.D.P. Wootton).
   - Stockholm (Sweden), Karolinska Sjukhuset (B. Thorell).

3. British Drug Houses Ltd.

4. Diagnostic Reagents Ltd., 60 North Street, Thame, Oxon.
International Committee for Standardization in Haematology of the European Society of Haematology  355

2.3 Measurement in near infra red to check turbidity (between 670 and 800, e.g., \( \lambda = 750 \) nm). The value should be less than 0.002.

3 STABILITY The standard is kept at 4°C and room temperature. Its stability is controlled by repeating the purity controls at three-monthly intervals.

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

REFERENCES

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