International standard for haemoglobinometry¹

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In August 1966, in Sydney, a General Assembly of the International Committee for Standardization in Haematology (I.C.S.H.) gave final approval to the Committee's recommendations on the use of a solution of cyanmethaemoglobin as a haemoglobin standard, on methods of haemoglobinometry on the basis of this standard, and on the specifications for an I.C.S.H. reference standard. The British Committee for Standards in Haematology was represented at the Assembly. The recommendations have been incorporated into a British Standard (BS 3985) and the British Committee for Standards in Haematology has established a national scheme for controlling and certifying standard solutions of cyanmethaemoglobin, including direct comparison with the International Reference Standard, to ensure that they conform to the I.C.S.H. specifications.

I RECOMMENDED METHOD

I 1 PRINCIPLE Haemoglobin is a chromoprotein. 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 molecular weight (relative molecular mass) of 64,458 (anhydrous) (Braunitzer, Gehring-Müller, Hilschmann, Hille, Hobom, Rudolf, and Wittmann-Liebold, 1961; Braunitzer, 1964; Hill, Konigsberg *et al.*, 1962). The iron concentration is, then, 0.347% (w/w) (mass fraction 0.00347). At present, data concerning the haemoglobin content of blood are expressed, for clinical purposes, in grams per 100 ml.²

It is recommended that the cyanmethaemoglobin method be used for clinical haemoglobinometry. If any other method is used (*e.g.*, photometric determination of oxyhaemoglobin, iron determination, gas analytic methods) it should be adjusted to obtain results which can be compared with those of the cyanmethaemoglobin method. The acid-haematin method is inaccurate and should not be used.

I 2 REAGENT The haemoglobin derivatives existing in blood, with the exception of verdoglobin (sulphaemoglobin), are converted into cyanmethaemoglobin by the use of an appropriate reagent. This must be of such a quality that after dilution of the blood there is no turbidity.³ To assure complete conversion the photometric determination must be delayed until the reaction is completed.

¹This report is based on the official I.C.S.H. *Recommendations for Haemoglobinometry in Human Blood*, as published in *Brit. J. Haemat.* (1967). 13 (Supplement pp. 71-75).

²The commission of Clinical Chemistry of the International Union of Pure and Applied Chemistry (I.U.P.A.C.) has recommended that the concentration be expressed as the molecular concentration in millimoles per litre, indicating the formula unit employed by the use of the notation $\frac{1}{2}\text{Hb}_2$ or (Fe).

I 3 EXTINCTION MEASUREMENT When a spectrophotometer is used the blood should be diluted suitably (*e.g.*, 1 : 251) with the reagent and measured at 540 m μ (or, with a mercury lamp, at the mercury line 546 m μ).

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

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.

In each case the instrument must be calibrated by means of a standard solution. Even minor changes in the set up of the method may cause significant deviations in calibration. (For details of method of using the standard in haemoglobin determination (see Lewis, 1967).)

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

I 4 STANDARD The cyanmethaemoglobin standard solution should be an aqueous solution of cyanmethaemoglobin with a concentration in the range of 55 to 85 mg. per 100 ml.⁴ It is strongly recommended that it be dispensed as a sterile solution in individual doses in sealed amber glass ampoules.

The spectrophotometric characteristics must conform to the same specifications as the I.C.S.H. cyanmethaemoglobin Reference Standard.

II I.C.S.H. CYANMETHAEMOGLOBIN (HAEMIGLOBINCYANIDE) REFERENCE STANDARD

II 1 MANUFACTURE The reference standard is prepared on behalf of the Committee by the Rijks Instituut voor de Volksgezondheid, Utrecht, Netherlands. Details of the method are given by Holtz (1965). The standard consists of washed human red cells, haemolysed by toluene and

³Drabkin's reagent consists of NaHCO₃, 1 g., K₃Fe(CN)₆, 200 mg., KCN 50 mg., and distilled water to 1 litre. It has a pH of 8.6; a modified reagent without the NaHCO₃ has a pH of 9.6. These solutions have a conversion time of 10 minutes or more. A suitable reagent with a shorter conversion time (recommended by van Kampen and Zijlstra, 1961) is prepared as follows: Dissolve 200 mg. of K₃Fe(CN)₆, 50 mg. of KCN, 140 mg. of KH₂PO₄ and an appropriate quantity of a non-ionic detergent in water and dilute to 1 litre. The pH should be 7.0 to 7.4 (pH meter). If stored at room temperature in a black-painted polyethylene 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: Sterox SE (concentrated), 0.5 ml./l., available from Hartman-Leddon Company, Philadelphia, U.S.A., or its representatives; Nonidet P40, 1 ml./l., available from Shell International Chemical Company.

⁴Solutions of lower concentration are not reliable.

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.⁵

II 2 EVALUATION AND CONTROL Each batch is tested in five laboratories nominated by the Committee,⁶ in accordance with the following principles:—

II 2 1 CONTENT The haemoglobin content is calculated from:—

$$c \text{ (mg. per 100 ml.)} = \frac{D_{\text{HICN}}^{540} \times 64,500}{44.0 \times d \times 10} = 146.5 \times D,$$

where: D_{HICN}^{540} = optical density of the solution at $\lambda = 540 \text{ m}\mu$,
 64,500 = molecular weight of haemoglobin (derived from 64,458),
 44.0 = $\epsilon_{\text{HICN}}^{540}$ (millimolar extinction coefficient)⁷,
 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_{HICN}^{540} is measured on a spectrophotometer, the wavelength scale of which has been calibrated with the aid of the Hg (of H) emission spectrum and absorption checks have been performed.⁸ Its slit width is so chosen that the half intensity band-width is less than $1 \text{ m}\mu$. The cuvettes in which the standard is measured are plan-parallel with an inner wall-to-wall distance of 1.000 cm. , tolerance of 0.5% (0.995 to 1.005). The measurements are carried out at 20° to 25°C.

The results are correlated at the Rijks Instituut voor de Volksgezondheid. The statistical mean of the results from the five laboratories is recorded; erratic results are discarded in accordance with current statistical practice. Experience with this procedure to date has shown that the confidence limits of the final results are within $\pm 1\%$.

II 2 2 PURITY The purity is controlled by:—

II 2 2 1 Judging the shape of a λD curve between $\lambda = 450 \text{ m}\mu$ and $\lambda = 700 \text{ m}\mu$, layer thickness 1.000 cm. ⁹

II 2 2 2 Determination of the quotient

$$\frac{D_{\text{HICN}}^{540}}{D_{\text{HICN}}^{504}}$$

⁵It was agreed that whereas the international reference solution would be aqueous, national boards might provide glycerinated solutions if preferred.

⁶At present these laboratories are:
 Cleveland (U.S.A.), Reference Laboratory of the College of American Pathologists, Cleveland Clinic (J. W. King).
 Freiburg i. Br. (Germany), Medizinische Universitätsklinik (A. von Klein-Wisenberg).
 Groningen (Netherlands), Fysiologisch Laboratorium Rijks Universiteit (W. G. Zijlstra) and Diaconessenhuis (E. J. van Kampen).
 London (United Kingdom), Royal Postgraduate Medical School (I. D. P. Wootton).
 Stockholm (Sweden), Karolinska Sjukhuset (B. Thorell).

⁷See Zijlstra and van Kampen (1960).

⁸See Menzies (1960).

⁹See van Kampen, Zijlstra, van Assendelft, and Reinkingh (1965).

The value of this quotient should lie between 1.59 and 1.63 .

II 2 2 3 Measurement in near infrared to check turbidity (between $\lambda = 670$ and $800 \text{ m}\mu$, e.g., at $\lambda = 750 \text{ m}\mu$). The optical density should be less than 0.002 per cm. pathlength.

II 2 3 STABILITY The standard is kept at 4°C. and at room temperature. Its stability is controlled by the testing laboratories repeating the primary standardization several times per year. The manufacturing laboratory repeats absorption measurements at more frequent intervals.

II 2 4 STERILITY¹⁰ 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°C. and 37°C.

II 3 LABELLING The standard is labelled with the batch number, the value of its haemoglobin content (mean and standard deviation, see section II 2 1) and an expiry date which is considered well within safe limits. The producer notifies all consumers if continuing control of stability indicates that a particular batch is no longer acceptable.

II 4 DISTRIBUTION¹¹ The standard is made available for reference use only to national standard committees for haematological methods or to official government-nominated holders.

The national holder must ensure that an opportunity is given to manufacturers and distributors to use the international standard as a reference standard if so desired, in conformity with national requirements.

Information regarding the availability of the I.C.S.H. Reference Standard in Britain, and the certification scheme of the British Committee for Standards in Haematology may be obtained from Dr. S. M. Lewis at Royal Postgraduate Medical School, Ducane Road, London, W.12.

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¹⁰Optional but always checked by the producer.

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