

Leaders

Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1995) and specifications for international haemoglobinocyanide standard (4th edition)

International Council for Standardisation in Haematology: Expert Panel on Haemoglobinometry

Introduction

Scientific symposia on haemoglobinometry were held at the 9th Congress of the European Society of Haematology, Lisbon, 1963,¹ and the 10th Congress of the International Society of Haematology (ISH), Stockholm, 1964.² The International Committee (now Council) for Standardisation in Haematology (ICSH) made recommendations endorsed by the General Assembly of ICSH in Sydney on 23 August 1966,³ for a reference method for haemoglobinometry and for the manufacture and distribution of an international reference preparation. Further symposia were held at the 12th Congress of the ISH, New York, 1968,⁴ and at the 13th Congress of the ISH, Munich, 1970.⁵ The recommendations were reissued in 1978⁶ and in 1986.⁷ On the basis of continuing experimental studies, the reference method and the specifications for the international standard have been modified. The revised recommendations are described in this document.

Recommendations for reference method for haemoglobinometry in human blood

Haemoglobin is a chromoprotein. On the basis of the chemical structure 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).⁸⁻¹⁰ The mass fraction of haemoglobin iron is therefore 0.003 47.

At the Assemblies in Kyoto, Japan, in 1976 and Budapest, Hungary, in 1984, ICSH recommended that haemoglobin in blood should be expressed as mass concentration in g/l. In conformity with the joint recommendation for use of the international system of units (SI) in clinical laboratory measurements, as agreed by ICSH, the International Federation of Clinical Chemistry (IFCC), and the World Association of Societies of Pathology (WASP),¹¹ substance concentration (mmol/l) may also be used. In that case the elementary entity (monomer or tetramer) should be specified by use of the

notation Hb(Fe) or Hb(4Fe). For measurement of the reference standard the expert panel recommends relating absorptivity and relative molecular mass to one haem group and one quarter of the total globin moiety.

PRINCIPLE

Photometric determination of haemoglobinocyanide (HiCN) (see note 1) is recommended as the reference method. If any other method is used in routine measurement (for example, photometric determination of oxyhaemoglobin or haemoglobinazide; iron determination) it should be adjusted to obtain comparability with the haemoglobinocyanide method.¹²

REAGENT

The haemoglobin derivatives occurring in blood, with the exception of sulphaemoglobin (see note 2), are converted into haemoglobinocyanide by the use of an appropriate reagent. The reagent must be of such quality that after dilution of the blood there is no turbidity (see note 3); the photometric determination must be delayed until the reaction is completed.

ABSORBANCE MEASUREMENT

Blood should be diluted suitably (for example, 1:251; 100 μ l of blood + 25 ml reagent)^{13 14} with reagent, filtered with a low-binding, low-release membrane filter, 0.2 to 0.25 μ m mean pore diameter (see note 4) to ensure absence of all particulate matter, and measured at 540 nm (see note 5) against an appropriate blank (see note 6).

The photometer must be calibrated as to wavelength and should be checked frequently as to linearity (see note 7) and the absence of stray light; the absorbance scale should be checked before each measurement series with— for example, a certified glass filter, or a solution

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of known absorbance—for example, a secondary haemoglobin standard. Even minor changes in the set-up may cause significant deviations in calibration.

CALIBRATION STANDARD

The haemoglobin cyanide calibration standard should be an aqueous solution of haemoglobin cyanide with a concentration in the range 550–850 mg/l (see note 8). It is strongly recommended that it be dispensed as a sterile solution in individual doses in sealed glass ampoules. The spectrophotometric characteristics must conform to the specifications of the International Haemoglobin cyanide Standard: $1.59 \leq A_{\text{HiCN}}^{540}/A_{\text{HiCN}}^{504} \leq 1.63$; $A_{\text{HiCN}}^{750} \leq 0.003$ per cm lightpath length; and an absorbance spectrum characteristic for pure HiCN solutions.¹⁵

DETERMINATION OF HAEMOGLOBIN

CONCENTRATION

Because HiCN solutions strictly follow Lambert–Beer's law, the haemoglobin concentration is calculated using the relation between absorbance and HiCN concentration established in the calibration procedure. If this relation is found to be in conformity with $\epsilon = 11.0 \text{ l.mmol}^{-1}.\text{cm}^{-1}$, the haemoglobin concentration is calculated by means of the following equation:

$$c(\text{g/l}) = \frac{A_{\text{HiCN}}^{540} \times M \times F}{\epsilon_{\text{HiCN}}^{540} \times 1 \times 1000} \quad (1)$$

where A_{HiCN}^{540} = absorbance of the solution at $\lambda = 540 \text{ nm}$; M = relative molecular mass of haemoglobin, derived from $64 \ 458/4^9$; F = dilution factor used (for example, 251); $\epsilon_{\text{HiCN}}^{540}$ = millimolar absorptivity = $11.0^{17,18}$; l = lightpath in cm; and 1000 = conversion factor mg to g. For a dilution (F) of 251 and a lightpath of 1.000 cm:

$$c(\text{g/l}) = 367.7 \times A_{\text{HiCN}}^{540} \quad (2)$$

International haemoglobin cyanide standard

MANUFACTURE

The international haemoglobin cyanide standard (see note 9) is manufactured at three to five year intervals; details of the method are given by Holtz.¹⁹ It is made from washed red cells from human blood tested for the absence of hepatitis and HIV antibodies, haemolysed by toluene, and centrifuged-free from debris. The haemoglobin is converted to haemoglobin cyanide. The final solution has a haemoglobin cyanide concentration of 550–850 mg/l. It is dispensed in sealed 10 ml glass ampoules as a sterile solution after membrane filtration (see note 10).

EVALUATION AND CONTROL

Each batch is tested in laboratories nominated by the ICSH Board (see note 11), 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.

CONCENTRATION

The HiCN concentration is calculated from the results of at least five laboratories, using the equation:

$$c(\text{g/l}) = \frac{A_{\text{HiCN}}^{540} \times 16 \ 114.5}{11.0 \times 1.000} = 1465 \times A_{\text{HiCN}}^{540}$$

where A_{HiCN}^{540} = absorbance of the solution at $\lambda = 540 \text{ nm}$; 16 114.5 = relative molecular mass of haemoglobin monomer, derived from $64 \ 458/4^9$; 11.0 = $\epsilon_{\text{HiCN}}^{540}$ (millimolar absorptivity) (see note 12); 1.000 = lightpath in cm.

An equivalent haemoglobin concentration of the standard may be calculated by multiplying the HiCN concentration by the dilution factor (F) used in the method—for example, 251 for 100 μl blood plus 25 ml reagent.

Using an appropriate blank (see note 6), A_{HiCN}^{540} is measured on a spectrophotometer, the wavelength scale of which has been calibrated with the aid of the mercury (or hydrogen or deuterium) emission spectrum, or holmium oxide in perchloric acid^{20,21}; on which absorption checks have been performed using calibrated glass filters or other means which have been certified by a standardising authority (see note 13); and of which the absence of stray light has been verified. Its slit width is chosen so that the half intensity bandwidth is less than or equal to 2 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 arithmetical mean and standard error of the results of the laboratories are recorded after erratic results, if any, have been discarded in accordance with statistical practice.²² Experience with this procedure has shown that the confidence limits of the final results are within $\pm 1\%$.

PURITY

The purity is controlled by (1) judging the shape of the absorbance curve between $\lambda = 450$ and 750 nm, lightpath length 1.000 cm (see note 14); (2) determining the quotient $A_{\text{HiCN}}^{540}/A_{\text{HiCN}}^{504}$, the value of this quotient should lie between 1.59 and 1.63; and (3) measuring at a wavelength between 730 and 770 nm (near-infrared) to check turbidity. This absorbance should be ≤ 0.003 per cm lightpath length, using an appropriate blank (see note 6).

STABILITY

The stability is checked by ICSH testing laboratories several times each year. The manufacturing laboratory repeats absorbance measurements at more frequent, regular intervals. The International Reference Standard is kept at 4°C. It is stable for more than six years.^{15,23}

STERILITY

In conformity with current practice of sterility control, the contents of the ampoules to be tested are innoculated in aerobic and anaerobic media and incubated at 22 and 37°C (see note 15).

LABELLING

The International Standard is labelled with the batch number, the value of its haemiglobincyanide concentration (mean and standard error; see concentration), and an expiry date which is considered well within safe limits (see stability).

The producer notifies all customers if continuing control of stability indicates that a particular batch is no longer acceptable.

PURPOSE AND DISTRIBUTION

The ICSH International Standard is used to assign values to secondary HiCN reference solutions or HiCN calibration solutions, and as a reference against which to judge the purity of such HiCN solutions.

The International Standard is made available for reference use only to national standards committees for haematological methods or to official government nominated holders (see note 16). Where there is no committee or official holder, it may be distributed to individuals approved by ICSH.

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

Information about national committees, official holders, and other contact persons may be obtained from the ICSH Secretariat, c/o Dr R M Rowan, Haematology Department, Western Infirmary, Dumbarton Road, Glasgow G11 6NT.

Notes

(1) Alternative terms for haemiglobincyanide are cyanmethaemoglobin and cyanferrihaemoglobin.

(2) Sulphaemoglobin is converted to sulphaemiglobincyanide. It has, however been calculated that the presence of 1% sulphaemoglobin in a blood specimen causes the total haemoglobin concentration to be underestimated by no more than 0.27%²⁴; in clinical practice sulphaemoglobin fractions exceeding 0.05 are seldom encountered.

(3) A suitable reagent, recommended by van Kampen and Zijlstra,²⁵ is prepared as follows: dissolve 200 mg $K_3Fe(CN)_6$, 50 mg KCN, 140 mg KN_2PO_4 (analytical grade chemicals), and an appropriate amount of non-ionic detergent in water and dilute to 1 litre. The pH must be 7.0–7.4; the osmolality 6–7 mmol/kg. The absorbance at $\lambda = 540$ nm, measured against water as blank, should be zero. If stored at room temperature in a brown borosilicate glass bottle, the solution keeps for at least two

months at approximately 20°C. $K_3Fe(CN)_6$ is destroyed by freezing; this decomposition can be prevented by the addition of ethanol, methanol, ethylene glycol (20 ml/l), or glycerol (5 ml/l).²⁶ Examples of suitable non-ionic detergents are Nonidet P40 (Shell International) 1 ml/l and Triton X-100 (Rohm and Haas) 1 ml/l. They are available in general from laboratory chemical suppliers. The reagent has a conversion time of three to five minutes in contrast to the original or modified Drabkin's reagent with a conversion time of 15 minutes or more. Because of the longer conversion time, as well as the greater incidence of turbidity, these other reagents are not recommended. A modified van Kampen-Zijlstra reagent has been described²⁷ for suppression of turbidity in samples from patients with a high white blood cell count or a high γ globulin concentration. The conversion time of carboxyhaemoglobin to HiCN is considerably longer, up to 30 minutes.¹⁵

(4) Examples of low-binding, low-release filters are (0.22 μ m) polyvinylidene difluoride Millex-GV (Millipore) and (0.20 μ m) polypropylene Paradisc 25 PP (Whatman). (B. Houwen, Loma Linda University Medical School: personal communication).

(5) High resolution spectrophotometry by the panel and by the US National Institute of Standards and Technology has shown the wavelength of maximal absorbance to be 541.05 ± 0.11 nm, and an absorbance minimum to be at 503.47 ± 0.14 nm. When measuring at 540 nm, the absorbance difference with respect to 541.05 nm is 0.1%.

(6) The reagent does not absorb light (see note 3). Some modern spectrophotometers have, however, been identified that show apparent light absorption between 500 and 600 nm by the reagent. It is not clear whether this apparent absorption is caused by stray light or by internal cuvette reflections. The laboratory should thus verify that, for a particular instrument-cuvette combination, the reagent does not absorb light. If apparent light absorption is found, a reagent blank should be used when measuring.

(7) Linearity may be verified using a procedure such as that described by Eilers and Crocker²⁸ with a sterile, membrane-filtered (at pore size 0.2–0.25 μ m) haemiglobincyanide calibration standard. With present-day spectrophotometers, calibration may resolve itself into verifying that the instrument yields an accurate value for A_{HiCN}^{540} of a secondary haemiglobincyanide standard. Slight deviations from the expected A_{HiCN}^{540} value may be used to correct measurement results.

(8) Solutions of lower concentration are not reliable.

(9) The standards were originally designated by the World Health Organisation (WHO)²⁹ as International Haemiglobincyanide Reference Preparations. They are now designated International Haemiglobincyanide Standards.

(10) It was agreed that, whereas the International Reference Preparation would be aqueous, national boards might provide glycerinated solutions if preferred.

- (11) At present these laboratories are Atlanta (USA), Scientific Resources Program, NCID, Centers for Disease Control and Prevention (O W van Assendelft); Emmen (The Netherlands), Scheperziekenhuis (A Zwart); Gaithersburg (USA), Laboratory of the College of American Pathologists, National Institute of Standards and Technology (M Welch); Groningen (The Netherlands), University of Groningen, Faculty of Medicine (W G Zijlstra); Loma Linda (USA), Loma Linda University Medical School (B Houwen); London (UK), Royal Postgraduate Medical School (S M Lewis); Rome (Italy), Istituto Superiore di Sanita (A M Salvati).
- (12) See van Kampen and Zijlstra¹⁵, Zijlstra and van Kampen¹⁷, and van Assendelft and Zijlstra.¹⁸
- (13) See Menzies³⁰ and National Bureau of Standards.³¹
- (14) See van Kampen *et al*¹³ and van Kampen and Zijlstra.¹⁵
- (15) Always checked by the producer.
- (16) The distribution is sponsored by the WHO.²⁹
- 1 ESH. Haemoglobinometry: erythrocytometric methods and their standardization. *Bibl Haematol* 1964;18:59-85.
 - 2 ISH. Haemoglobinometry and molecular weight of haemoglobin. Standardization, documentation and normal values in haematology. *Bibl Haematol* 1965;21:25-102.
 - 3 ICSH. Recommendations for haemoglobinometry in human blood. *Br J Haematol* 1967;13(Suppl):71-6.
 - 4 Astaldi G, Sirtori C, Vanzetti G (eds). *Standardization in haematology*. Milan: Fondazione Carlo Erba, 1969.
 - 5 Izak G, Lewis SM (eds). *Modern concepts in haematology*. New York: Academic Press, 1972.
 - 6 ICSH. Recommendations for reference method for haemoglobinometry in human blood (ICSH Standard EP6/2: 1977) and specification for international haemoglobinocyanide reference preparation (ICSH Standard EP6/3:1977). *J Clin Pathol* 1978;31:139-43.
 - 7 ICSH. Recommendations for reference method for haemoglobinometry in human blood (ICSH standard 1986) and specifications for international haemoglobinocyanide reference preparation (3rd edition). *Clin Lab Haematol* 1987;9:73-9.
 - 8 Braunitzer G, Gehring-Muller R, Hilschmann N, Hilde K, Hobom G, Rudloff V, *et al*. Die Konstitution des normalen adulten Humanhämoglobins. *Hoppe-Seylers Zeitschrift für physiologische Chemie* 1961;325:283-6.
 - 9 Braunitzer G. The molecular weight of haemoglobin. *Bibl Haematol* 1964;18:59-60.
 - 10 Hill RJ, Konigsberg W, Guidotti G, Craig LC. The structure of human hemoglobin. Parts 1-5. *J Biol Chem* 1962;237:1549-54;2184-95;2547-61;3151-6;3157-62.
 - 11 ICSH, IFCC, WASP. Recommendations for use of SI in clinical laboratory measurements. *Br J Haematol* 1972;23:787; *Z Klin Chem Klin Biochem* 1973;11:93.
 - 12 Kwant G, Oeseburg B, Zwart A, Zijlstra WG. Calibration of a practical haemoglobinometer. *Clin Lab Haematol* 1987;9:387-93.
 - 13 van Kampen EJ, Zijlstra WG, van Assendelft OW, Reinkingh WH. Determination of hemoglobin and its derivatives. *Adv Clin Chem* 1965;8:141-87.
 - 14 van Assendelft OW. *Spectrophotometry of haemoglobin derivatives*. Assen: van Gorcum, 1970; Springfield, Illinois: Charles C Thomas, 1970.
 - 15 van Kampen EJ, Zijlstra WG. Spectrophotometry of hemoglobin and hemoglobin derivatives. *Adv Clin Chem* 1983;23:199-257.
 - 16 Zijlstra WG, Buursma A, Zwart A. Molar absorptivities of human hemoglobin in the visible spectral range. *J Appl Physiol* 1983;54:1287-91.
 - 17 Zijlstra WG, van Kampen EJ. Standardization of hemoglobinometry. I. The extinction coefficient of hemoglobinocyanide at $\lambda = 540 \text{ nm}$: $\epsilon_{\text{HbCN}}^{540}$. *Clin Chim Acta* 1960;5:719-26.
 - 18 van Assendelft OW, Zijlstra WG. Extinction coefficients for use in equations for the spectrophotometric analysis of haemoglobin mixtures. *Anal Biochem* 1975;69:43-8.
 - 19 Holtz AH. Some experience with a cyanhemoglobin solution. *Bibl Haematol* 1965;21:75-8.
 - 20 Jansen AP, van Kampen EJ, Steigstra H, van der Ploeg PHW, Zwart A. Simultaneous spectrophotometric calibration of wavelength and absorbance in an interlaboratory survey using holmium oxide (Ho₂O₃) in perchloric acid as reference, compared with p-nitrophenol and cobaltous sulfate solutions (1978-1984). *J Clin Chem Clin Biochem* 1986;24:141-6.
 - 21 Weidner VR, Mavrodineanu R, Mielenz KD, Velapoldi RA, Eckerle KL, Adams B. Spectral transmittance characteristics of holmium oxide in perchloric acid solution. *Journal of Research of the National Bureau of Standards* 1985;90:115-25.
 - 22 Dixon WJ. Processing data for outliers. *Biometrics* 1953;9:74-89.
 - 23 van Assendelft OW, Buursma A, Holtz AH, van Kampen EJ, Zijlstra WG. Quality control in haemoglobinometry with special reference to the stability of haemoglobinocyanide reference solutions. *Clin Chim Acta* 1976;70:161-9.
 - 24 Dijkhuizen P, Buursma A, Gerding AM, Zijlstra WG. Sulphaemoglobin. Absorption spectrum, millimolar extinction coefficient at $\lambda = 620 \text{ nm}$, and interference with the determination of haemoglobin and of haemoglobinocyanide. *Clin Chim Acta* 1977;78:479-87.
 - 25 van Kampen EJ, Zijlstra WG. Standardization of hemoglobinometry. II. The hemoglobinocyanide method. *Clin Chim Acta* 1961;6:538-44.
 - 26 Zweens J, Frankena H, Zijlstra WG. Decomposition on freezing of reagents used in the ICSH-recommended method for the determination of total haemoglobin in blood, its nature, cause, and prevention. *Clin Chim Acta* 1979;91:337-52.
 - 27 Matsubara T, Okuzono H, Senba U. A modification of van Kampen-Zijlstra's reagent for the hemoglobinocyanide method. *Clin Chim Acta* 1979;93:163-4.
 - 28 Eilers R, Crocker C. The value of Ringbom curves in hemoglobinometry. In: Izak G, Lewis SM, eds. *Modern concepts in hematology*. New York: Academic Press, 1972: 58-65.
 - 29 World Health Organization. WHO Expert Committee on Biological Standardization. 20th Report. *World Health Organ Tech Rep Ser* 1968;384:12-13;86-7.
 - 30 Menzies AC. Report on calibration of wavelength and photometric scales of non-recording spectrophotometers. *Pure Appl Chem* 1960;1:147-62.
 - 31 National Bureau of Standards. Standard reference materials No. 51: Glass filters as a standard reference material for spectrophotometry. NBS Special Publication 260. Washington DC: Government Printing Office, 1975.