Control data of international haemiglobincyanide reference solutions

At its 1964 Stockholm meeting the International Committee for Standardization in Haematology (ICSH) instituted an Expert Panel on Haemoglobinometry to advise the ICSH board on proposals for an internationally acceptable standardized method for the determination of the haemoglobin content of human blood. The panel would also name a number of reference laboratories to determine the haemoglobin content of international haemiglobincyanide (HiCN) reference solutions prepared by the Netherlands Institute of Public Health[1]. During the period of issue of the reference solution the reference laboratories were also to perform content and quality checking. This paper deals with the measurement results of the first two batches of HiCN reference solution.

METHODS

The HiCN reference solutions were prepared according to Zijlstra and Van Kampen[2]. Content and purity of the HiCN solutions were determined and checked by laboratories[3] in the U.S.A., Great Britain, Germany, Sweden and the Netherlands. The haemoglobin content was calculated from spectrophotometric data using the equation

$$c = \frac{D \times 64458}{44.0 \times l \times 10}$$

where c = haemoglobin content in mg per 100 ml; D = optical density of the HiCN solution at λ = 540 nm; 64458 = molecular weight of haemoglobin[4]; 44.0 = millimolar extinction coefficient of HiCN at λ = 540 nm (ref. 5); l = light path length; 10 = conversion factor from l to 100 ml.

The purity of the HiCN solutions was determined by judging the shape of the absorption spectrum by measuring the quotient D^{540}/D^{504} and by measuring D^{750}. The value for D^{540}/D^{504} should lie between 1.59 and 1.63, the value for D^{750} should be equal to or less than 0.002 per cm light path length. As HiCN does not absorb light at λ = 750 nm, measurement of D^{750} was solely meant as a check on turbidity of the solutions.

The international reference solution was at first checked every six months. During the first year all spectrophotometric readings were collected and the arithmetical mean calculated. This however gave rise to a very large number of sometimes diverging results. The expert panel therefore decided to partly standardize the measuring procedure in the reference laboratories. Each laboratory was asked to measure at least two ampoules of the standard every three months, preferably using two different non-recording spectrophotometers, the measurements being performed by two independent operators on each of the instruments. At the same time a spectrophotometric reading was defined as the result of as many separate readings the operator thinks necessary to obtain a stable value. At this time a standardized sheet was introduced to collect the following data: date of measurement; batch No.; laboratory and operator; make, type and serial No. of spectrophotometer used; method of wavelength calibration; method of optical density check; half-intensity band width of measuring beam at λ = 750, 540 and 504 nm; inner wall-to-wall distance of the

cuvettes; sample temperature; blank against which the solutions are measured; optical density reading of samples measured at $\lambda = 750$, 540 and 504 nm.

The spectrophotometers themselves were checked regularly as to wavelength by means of the mercury emission lines at $\lambda = 546.1$ and 491.6 nm. Two laboratories used the hydrogen emission lines at $\lambda = 656.3$ and 486.1 nm. The optical density scale was checked at $\lambda = 540$ nm by means of a Corning HT yellow glass filter certified by the American National Bureau of Standards. The shape of the density/wavelength curve of this filter is given in Fig. 1. Although objections may of course be raised
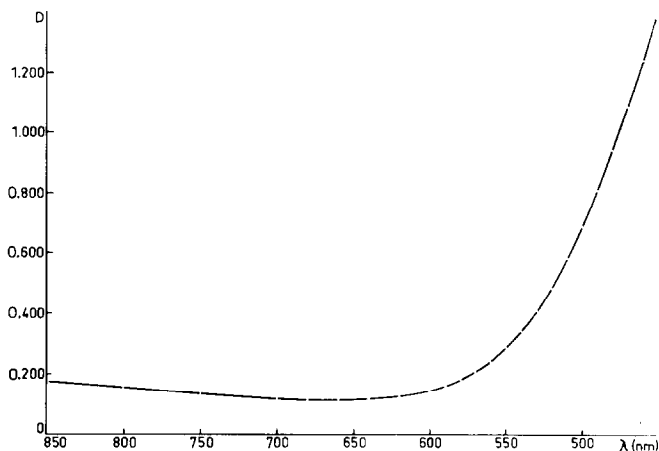

Fig. 1. Optical density–wavelength curve of Corning HT yellow filter. Optica CF4 spectrophotometer.

against measuring on the rather steep part of this curve as an optical density check, very consistent results have been obtained in the laboratories. As yet, no other acceptable stable glass filter has been encountered for optical density calibration purposes. Experiments using neutral density and didymium glass filters are, however, being performed.

RESULTS AND DISCUSSION

Table I shows the mean of all the measurements at $\lambda = 540$ nm performed in each of the laboratories separately at a given time. The number of measurements and the value for D^{540}/D^{504} are also included. The D^{750} measurements have not been included as they were nearly always found to lie between 0.000 and 0.002 per cm light path length.

Starting March 1966, each laboratory used the standardized data sheet. The first reference solution (batch 40400) was issued for a period of two years, the second (batch 60400) was first checked for nine months and issued for use in January 1967, at which time the first solution was declared non-valid.

Table II shows the mean of all measurements performed at $\lambda = 540$ and 504 nm by all the laboratories. Standard deviation and standard error of the mean have also been included. In one case, only the mean value of 10 measurements was sent

TABLE I

D^{540} AND D^{540}/D^{504} VALUES OF INTERNATIONAL HiCN REFERENCE SOLUTIONS AS MEASURED IN THE DIFFERE
Batch 40400

Laboratory	Nov. 1964			June 1965			Nov. 1965			March 1966			June 1966		
	D^{540}	$\dfrac{D^{540}}{D^{504}}$	n	D^{540}	$\dfrac{D^{540}}{D^{504}}$	n	D^{540}	$\dfrac{D^{540}}{D^{504}}$	n	D^{540}	$\dfrac{D^{540}}{D^{504}}$	n	D^{540}	$\dfrac{D^{540}}{D^{504}}$	n
1	0.387	1.61	9	0.385	1.61	9	0.387	1.62	12	0.389	1.61	3	0.388	1.61	3
2	0.394	1.61	9	0.391	1.61	33	0.391	1.61	9	0.392	1.60	6	0.386	1.61	18
3	0.391	1.61	21	0.390	1.61	15	0.391	1.61	27	0.391	1.61	18	0.390	1.61	14
4	0.391	1.61	9	0.395	1.62	10	0.390	1.60	9	0.386	1.63	3	0.388	1.60	3
5	0.391	1.61	18	0.390	1.60	27	0.392	1.61	21	0.390	1.61	12	0.390	1.59	12
6	0.390	1.61	9	0.387	1.63	9	0.390	1.61	9	0.3895	1.61	6	0.3905	1.60	2

in by one of the laboratories. For the calculation of standard deviation and standard error of the mean, this mean value was taken as a single reading. The results are quite consistent during the period presented, again confirming the stability of the haemoglobin derivative used[6]. It is interesting to note that, although the absolute standard deviation calculated for D^{540} and for D^{504} differ but little, a difference of about a factor 2 is encountered when the standard deviation is calculated as a percentage of the optical density. This could mean that it is more difficult to measure accurately at a wavelength of minimal absorption than at a wavelength of peak absorption.

Although it was expected that spectrophotometric procedural standardization would improve upon the divergence of the measurement results, this was not found to be the case. To gain further insight in these differences, further statistical analysis was performed. At this moment the only feasible analysis was calculation of mean, standard deviation and standard error of the mean for the groups of different makes of spectrophotometers used. As yet, insufficient data are available to allow accurate calculation for each laboratory and each instrument separately.

TABLE II

D^{540} AND D^{504} VALUES OF INTERNATIONAL HiCN REFERENCE SOLUTIONS
Mean value of laboratories 1–6.
Batch 40400

	D^{540}	s	$s/\sqrt{n}$	n	D^{504}	s	$s/\sqrt{n}$	n
Nov. 1964	0.391	0.0023	0.0003	75	0.243	0.0020	0.0002	75
June 1965	0.389	0.0026	0.0003	94	0.243	0.0036	0.0004	97
Nov. 1965	0.391	0.0022	0.0002	87	0.243	0.0020	0.0002	84
March 1966	0.390	0.0018	0.0003	48	0.242	0.0020	0.0003	48
June 1966	0.389	0.0025	0.0003	52	0.242	0.0031	0.0004	52
Sept. 1966	0.390	0.0020	0.0003	46	0.244	0.0029	0.0004	46
Nov. 1966	0.390	0.0026	0.0003	60	0.242	0.0033	0.0004	57
	Batch 60400							
June 1966	0.385	0.0026	0.0003	92	0.240	0.0027	0.0003	92
Sept. 1966	0.386	0.0023	0.0003	55	0.240	0.0026	0.0003	55
Nov. 1966	0.386	0.0033	0.0004	66	0.240	0.0028	0.0003	66
March 1967	0.385	0.0032	0.0004	53	0.240	0.0023	0.0003	53

Standard deviation $(s) = \sqrt{\dfrac{\Sigma(x-\bar{x})^2}{n-1}}$

Standard error of the mean $= s/\sqrt{n}$

LABORATORIES

Batch 60400

Sept. 1966			Nov. 1966			June 1966			Sept. 1966			Nov. 1966			March 1967		
D^{540}	$\dfrac{D^{540}}{D^{504}}$	n	D^{540}	$\dfrac{D^{540}}{D^{504}}$	n	D^{540}	$\dfrac{D^{540}}{D^{504}}$	n	D^{540}	$\dfrac{D^{540}}{D^{504}}$	n	D^{540}	$\dfrac{D^{540}}{D^{504}}$	n	D^{540}	$\dfrac{D^{540}}{D^{504}}$	n
0.387	1.60	3	0.389	1.59	3	0.386	1.60	3	0.387	1.60	3	0.380	1.62	3	0.387	1.60	3
0.389	1.57	12	0.388	1.58	15	0.384	1.61	18	0.386	1.58	12	0.386	1.60	15	0.383	1.62	18
0.390	1.61	10	0.3915	1.61	21	0.387	1.61	46	0.388	1.61	19	0.388	1.61	27	0.387	1.61	11
0.389	1.61	3	0.390	1.60	3	0.381	1.61	3	0.382	1.60	3	0.385	1.62	3	0.386	1.60	3
0.392	1.60	12	0.390	1.62	9	0.386	1.60	16	0.384	1.61	12	0.384	1.61	12	0.387	1.60	12
0.390	1.61	6	0.3895	1.60	6	0.384	1.60	6	0.385	1.61	6	0.386	1.60	6	0.3865	1.61	6

TABLE III

D^{540} AND D^{504} VALUES OF INTERNATIONAL HiCN REFERENCE SOLUTIONS AS MEASURED ON DIFFERENT SPECTROPHOTOMETERS

Batch 40400

Spectrophotometer	D^{540}	s	$s/\sqrt{n}$	n	D^{504}	s	$s/\sqrt{n}$	n
Zeiss PMQ II (2; 1)	0.389	0.0035	0.0004	78	0.244	0.0036	0.0004	78
Beckman DU (7; 5)	0.390	0.0024	0.00015	255	0.243	0.0024	0.0002	252
Optica CF4 (1; 1)	0.391	0.0011	0.0002	34	0.243	0.0017	0.0003	34
Unicam SP500 (2; 2)	0.390	0.0018	0.0002	89	0.243	0.0033	0.0003	89
Batch 60400								
Zeiss PMQ II (2; 1)	0.384	0.0040	0.0007	36	0.240	0.0046	0.0008	36
Beckman DU (7; 5)	0.386	0.0024	0.0002	127	0.240	0.0024	0.0002	127
Optica CF4 (1; 1)	0.387	0.0015	0.0002	41	0.240	0.0011	0.0002	41
Unicam SP500 (2; 2)	0.386	0.0018	0.0002	50	0.241	0.0017	0.0002	50

Standard deviation $(s) = \sqrt{\dfrac{\Sigma(x-\bar{x})^2}{n-1}}$ Standard error of the mean $= s/\sqrt{n}$

The first number in brackets (column one) refers to the number of separate instruments, the second to the number of laboratories using them.

The results are shown in Table III. One older instrument on which but 12 readings were taken, has not been included. It may possibly be inferred from these results that, even after calibration as to wavelength and as to optical density, the Zeiss instruments are not optimal for single point measurements.

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