

# Carboxyhaemoglobin: a possible reference material for haemoglobin assay

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**SUMMARY** The inter- and intralaboratory quality control of haemoglobin estimation in remote laboratories requires a more rugged control haemolysate than is commercially available. The stabilities of oxyhaemoglobin and carboxyhaemoglobin forms of an ethanediol-containing haemolysate were studied over a three-year period. From the results obtained, carboxyhaemoglobin under nitrogen is proposed as a possible candidate reference material for haemoglobin assay.

Van Assendelft *et al*<sup>1</sup> discussed an interlaboratory trial of quality control in haemoglobinometry and recommended that full intralaboratory control should incorporate the use of concentrated haemoglobin solutions, and that there should be frequent use of haemiglobincyanide (HiCN) reference solutions and checking the CN<sup>-</sup> content of the reagent.

They measured the haemoglobin content of a commercially available haemolysate (Merz and Dade AG, Bern, Switzerland), kept at 4°C over a period of 12 months, at monthly intervals, and using a new bottle of haemolysate for each measurement. During that time, which was the manufacturer's recommended period of stability, the haemoglobin content appeared to decrease by 0.2 g/dl.

Van Assendelft *et al*<sup>1</sup> stressed the need for concentrated haemoglobin haemolysates to be kept free from bacterial contamination and noted that the Merz and Dade haemolysate was membrane filtered to render it sterile. Lewis<sup>2</sup> has stated that the commercially available lysed blood standards tend to deteriorate during storage with the formation of haemiglobin (methaemoglobin) and denaturation of proteins; furthermore, repeated opening of the bottle for subsampling results in evaporation and contamination with micro-organisms. These observations have been endorsed by our own experience during the World Health Organisation (WHO)-sponsored interlaboratory trial of two types of solid state haemoglobinometers<sup>3</sup> designed for use in developing countries, when the need for a more rugged haemolysate for both intra- and interlaboratory quality control became apparent. Many laboratories, particularly in developing countries, are

at the end of very long and uncertain lines of communication<sup>4</sup> and quality control materials must be able to tolerate delays in transit at high temperatures.

The preparation of an ethanediol-containing haemolysate is described here, together with findings on the stability of its oxyhaemoglobin and carboxyhaemoglobin forms over a three-year period.

## Material and methods

Waring Blendor (Waring Products Div, Connecticut, USA).

Unicam SP 1800 dual beam spectrophotometer (Pye Unicam Ltd, Cambridge, UK).

Coulter counter S (Coulter Electronics Ltd, Luton, UK).

Hartridge reversion spectroscope (Ealing Beck Ltd, Watford, UK).

20 µl SwizzleStick.<sup>5</sup>

Dialysis tubing.

ICSH haemiglobincyanide reference preparation (batch no 50 500, Rijksinstituut voor de Volksgezondheid, Bilthoven, The Netherlands).<sup>6</sup>

ICSH Haemiglobincyanide diluent<sup>6</sup> (BDH, Poole, UK).

Isoton II and Lyse S (Coulter Electronics Ltd, Luton, UK).

Toluene and ethanediol (ethylene glycol), reagent grade (JT Baker, Phillipsburg, NJ, USA).

Oxygen and carbon monoxide, industrial grade (Matheson, Lyndhurst, NJ, USA).

Stock haemolysate, prepared May 1978, as follows: Cells from 1.5 l human blood were washed with 0.9% NaCl solution to remove plasma proteins. The washed, packed red cells were lysed by being stored at -20°C overnight and then thawed at 25°C. This

procedure was repeated two more times to ensure total haemolysis.

Toluene (40 ml) was added to each 100 ml of haemolysate and mixed (Waring Blendor) for two minutes. The mixture was centrifuged at 2075 *g* for 30 min, causing the cellular material to form a tight mat of precipitate between the aqueous haemolysate and the toluene. The toluene was discarded and the haemolysate filtered through glass wool; toluene was again added in the ratio of two volumes of toluene to five volumes of haemolysate. The solutions were mixed and centrifuged as before, the toluene discarded and the haemolysate again filtered through glass wool.

The haemolysate was concentrated in dialysis tubing in a stream of air from an electric fan for 4 h. This gave a solution with a haemoglobin concentration of 29 g/dl. The volume of concentrated haemolysate was measured and a half-volume of ethanediol was added to make a final solution which contained 33% vol/vol ethanediol.

#### *Oxyhaemoglobin*

Oxygen was bubbled slowly through a well-stirred portion of stock haemolysate solution for 30 min: 1.0 ml portion of the oxygenated solution were then dispensed into 5.0 ml clear soda glass vials. The contents were overlaid with oxygen, and the vials sealed and stored at  $-30^{\circ}\text{C}$ .

#### *Carboxyhaemoglobin*

Carbon monoxide was bubbled slowly through a portion of stirred stock haemolysate in an efficient fume cupboard for 30 min: 1.0 ml portion were dispensed into 5.0 ml clear glass ampoules, overlaid with nitrogen, sealed and stored at  $-30^{\circ}\text{C}$ .

### METHODS

#### *Experimental design*

Batches of 88 ampoules of carboxyhaemoglobin and 20 ampoules of oxyhaemoglobin were made available for long term stability testing. The ampoules were divided into four groups to be stored respectively at  $-30^{\circ}\text{C}$ ,  $4^{\circ}\text{C}$ , room temperature (RT  $11-26^{\circ}\text{C}$  over the trial period) and  $40^{\circ}\text{C}$ . Before being placed at these temperatures, the contents of half the ampoules of each group were transferred aseptically into sterile 5.0 ml bijoux bottles. There were two reasons for this manoeuvre:

- (1) to establish whether storage under gas in a sealed ampoule was really necessary for long-term stability;
  - (2) to establish whether subsampling caused evaporation losses or contamination by micro-organisms, or both.
- Being aware of the problems associated with sample

viscosity when using piston-operated air interface micropipettes,<sup>7</sup> the dilutions of haemolysate were conveniently made using a  $20\ \mu\text{l}$  positive displacement microlitre diluting device, the SwizzleStick.<sup>5</sup>

#### *Procedure*

The groups of ampoules and bottles were placed at their respective temperatures in May 1978. Visual inspection of ampoules and bottles after 24 h at  $40^{\circ}\text{C}$  revealed no obvious change, but after 72 h at  $40^{\circ}\text{C}$ , small "clots" had appeared in the ampoules and bottles of both the oxyhaemoglobin and carboxyhaemoglobin haemolysates; these were not investigated further.

The first haemoglobin measurements were made after three weeks. One ampoule and one bottle were selected at random from each of the  $-30^{\circ}\text{C}$ ,  $4^{\circ}\text{C}$  and RT groups. The ampoules were labelled HbCO 1A and the bottles, HbCO 1B, for each temperature. The  $-30^{\circ}\text{C}$  and  $4^{\circ}\text{C}$  ampoules were allowed to equilibrate to room temperature, when the contents of the ampoules from the three temperatures were transferred aseptically into sterile bijoux bottles, appropriately labelled. Dilutions (1/201) were made with haemiglobincyanide diluent and a period of two hours was allowed for complete conversion into haemiglobincyanide.<sup>8</sup> The absorbances of the diluted solutions and of the ICSH haemoglobin reference solution were measured at 540 nm and the haemoglobin content calculated. Once the dilutions had been made, the capped bottles with remaining contents were replaced at the appropriate temperature.

The second series of measurements was made after 12 wk. Bottles HbCO 1A and 1B from each group were re-examined, in conjunction with another ampoule, HbCO 2A and bottle, HbCO 2B from the  $-30^{\circ}\text{C}$ ,  $4^{\circ}\text{C}$  and RT groups. The procedure was the same as before, but the dilutions of the RT samples showed an insoluble granular precipitate and were unsuitable for absorbance measurements.

The third series of measurements took place at 38 wk. Bottles HbCO 1A, 1B, 2A and 2B from each group were re-examined, in conjunction with another ampoule, HbCO 3A and bottle, HbCO 3B from each of the  $-30^{\circ}\text{C}$ ,  $4^{\circ}\text{C}$  and RT groups. The RT samples were diluted and visually examined. As the insoluble granular precipitate was present in all of the tubes, their absorbances were not measured and all the ampoules and bottles being stored at RT were discarded.

The final series of measurements took place at 165 wk. All the remaining ampoules and bottles at  $-30^{\circ}\text{C}$  and  $4^{\circ}\text{C}$  that had not been previously sampled were examined, together with the remaining haemolysates of the earlier studies.

## Results

The results are shown in Table 1, each entry representing a single determination on material stored in the manner indicated.

### *Oxyhaemoglobin haemolysates*

The oxyhaemoglobin haemolysates were examined in the same way as the carboxyhaemoglobin samples but on three occasions only, at 8, 38 and 165 wk. On the second occasion, there was some precipitation when the RT samples were diluted. The results are shown in Table 2.

### *Presence of haem pigments*

When first sampled, all the ampoules and bottles were examined spectroscopically for the presence of haemoglobin. All the oxyhaemoglobin bottles and previously unopened vials which had been kept at  $-30^{\circ}\text{C}$ ,  $4^{\circ}\text{C}$  and RT and  $40^{\circ}\text{C}$  contained varying amounts of haemoglobin.

With the carboxyhaemoglobin series, haemoglobin could not be detected in the unopened vials with nitrogen overlay kept at  $40^{\circ}\text{C}$ , RT and  $4^{\circ}\text{C}$ , nor in the bottles and vials kept at  $-30^{\circ}\text{C}$  for up to 38 wk. The bottles at  $40^{\circ}\text{C}$ , RT and  $4^{\circ}\text{C}$  showed varying

amounts of haemoglobin. By 165 wk, haemoglobin was present in the previously unsampled bottles at  $-20^{\circ}\text{C}$  and  $4^{\circ}\text{C}$  but could not be detected in the unopened vials at  $-20^{\circ}\text{C}$  or  $4^{\circ}\text{C}$ .

Sulphaemoglobin was not detected spectroscopically in any of the foregoing samples but the possible presence of trace amounts of other pigments was not excluded.

### *Bacterial contamination*

When the final series of measurements was completed, the bottles that had been stored at  $-30^{\circ}\text{C}$  and  $4^{\circ}\text{C}$  were left at room temperature for 72 h. A  $10\ \mu\text{l}$  portion from each haemolysate was plated out on to blood agar and incubated at  $32^{\circ}\text{C}$  for 72 h. Bacterial growth did not occur on any of the plates.

## Discussion

The addition of ethanediol to the haemolysate, which is a simple and inexpensive procedure, evidently eliminated the need for membrane filtration or other special techniques designed to prevent bacterial contamination.

The oxyhaemoglobin haemolysate stored under oxygen at  $-30^{\circ}\text{C}$  and  $4^{\circ}\text{C}$  appeared to have reason-

Table 1 *Haemoglobin content (g/dl) of carboxyhaemoglobin haemolysate over 165-week period, measured as haemiglobincyanide*

Material	Period of storage (wk) and temperature										
	3			12		38		165			
	$-30^{\circ}\text{C}$	$4^{\circ}\text{C}$	RT	$-30^{\circ}\text{C}$	$4^{\circ}\text{C}$	$-30^{\circ}\text{C}$	$4^{\circ}\text{C}$	$-30^{\circ}\text{C}$	$4^{\circ}\text{C}$		
HbCO 1A (ampoule)	19.3	19.4	19.3	19.6	19.3	19.4	19.8	19.2		*NR	
1B (bottle)	19.3	19.3	19.3	19.3	19.3	19.3	19.9	19.7		*NR	
2A	—	—	—	19.4	19.4	19.3	19.3	19.0		19.6	
2B	—	—	—	19.6	19.3	19.3	19.5	19.7		20.0	
3A	—	—	—	—	—	19.3	19.2	19.1		19.7	
3B	—	—	—	—	—	19.3	19.2	19.8		19.4	
Remaining ampoules	—	—	—	—	—	—	—	19.1 (n = 4)		19.3 (n = 8)	
Remaining bottles	—	—	—	—	—	—	—	19.4 (n = 7)		19.3 (n = 8)	

\*No remaining sample.

Table 2 *Haemoglobin content (g/dl) of oxyhaemoglobin haemolysate over 165 week period, measured as haemiglobincyanide*

Material	Period of storage (wk) and temperature								
	8			38		165			
	$-30^{\circ}\text{C}$	$4^{\circ}\text{C}$	RT	$-30^{\circ}\text{C}$	$4^{\circ}\text{C}$	$-30^{\circ}\text{C}$	$4^{\circ}\text{C}$		
HbO <sub>2</sub> 1A (ampoule)	19.0	19.0	18.9	19.0	19.2	19.1		20.1	
1B (bottle)	19.0	18.7	18.7	19.1	19.0	19.1		19.7	
2A	—	—	—	19.1	19.2	19.1		19.4	
2B	—	—	—	19.4	19.2	19.1		18.9	
Remaining ampoules	—	—	—	—	—	*NR		19.25 (n = 2)	
Remaining bottles	—	—	—	—	—	*NR		19.55 (n = 2)	

\*No remaining samples.

able stability for at least three years when measured by the haemiglobincyanide method, but there was a trend towards an apparent small increase in haemoglobin concentration as judged by the known reproducibility of the method (0.7%);<sup>5</sup> this increase may have been an artefact of incipient turbidity. The increase was greater if air was not rigorously excluded by an oxygen overlay.

At room temperature (11-26°C), the preparation was stable for 8 wk, as measured with the haemiglobincyanide method but had deteriorated by 38 wk. At 40°C, the preparation was stable for 24 h but had deteriorated by 72 h.

The carboxyhaemoglobin haemolysate stored under nitrogen at -30°C or 4°C was found to be unconditionally stable for at least three years. When the nitrogen overlay was removed, haemoglobin formed and the haemoglobin concentration, as measured by the haemiglobincyanide method, apparently increased.

The carboxyhaemoglobin haemolysate with nitrogen overlay satisfied the criteria of Lewis,<sup>2</sup> as outlined above, for an ideal lysed blood reference material. It does have the disadvantage, when used with the ICSH diluent, of requiring two hours for complete conversion into haemiglobincyanide compared with a 3-5 min conversion time for blood samples which normally contain less than 3% carboxyhaemoglobin. However, complete conversion of the carboxyhaemoglobin haemolysate appears to take place in under one minute in the Coulter counter S when Isoton II and Lyse S are used as the diluent, but the kinetics of the conversion were not investigated.

The ICSH recommended diluent for haemiglobincyanide has been used extensively since it was proposed by van Kampen and Zijlstra in 1961.<sup>9</sup> However, shortcomings have been reported and various modifications suggested. Franzini *et al*<sup>10</sup> and Matsubara *et al*<sup>11</sup> proposed the addition of sodium chloride to reduce the incidence of turbidity due to abnormal plasma proteins and/or an increased number of circulating white blood cells. Zweens *et al*<sup>12</sup> examined the changes induced by freezing the reagent and proposed the addition of ethanol to extend the shelf life. Van Assendelft *et al*<sup>1</sup> acknowledged that the potassium cyanide in the reagent has poor long term stability and its concentration needs monitoring. The recommended conversion time of 3-5 min is in practice too long for both automated equipment and busy clinic estimations. A revised reagent, akin to the Coulter formulation in its speed of reaction, is now required.

In our judgement, the carboxyhaemoglobin

haemolysate under nitrogen would have a distinct advantage over commercial haemolysates for inter- and intralaboratory quality control where there are long or difficult lines of communication, provided that the material is not exposed to unduly high ambient temperatures for long periods in transit and that the importance of maintaining the integrity of the nitrogen overlay is recognised.

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