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Preparation and Control of Ethylene Glycol-Stabilized Haemolysates for Glycated Haemoglobin Assay

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Summary: The preparation and evaluation of ethylene glycol-stabilized haemolysates for use as control material for the assay of glycated haemoglobins is described. These haemolysates were prepared from normal and diabetic blood samples by following the procedure normally used to purify human haemoglobin, with the addition of dialysis to remove glucose from the labile fractions, and dilution with ethylene glycol. All the haemoglobin fractions were converted into the carbon monoxide form to increase their stability and were stored under different conditions.

During a 10 month period of storage at −20 °C no significant change in the glycated haemoglobins level was observed.

Bereitung und Prüfung von mit Ethylenglykol stabilisierten Hämolysaten zur Bestimmung glykosylierter Hämoglobine

Zusammenfassung: Bereitung und Bewertung von mit Ethylenglykol stabilisierten Hämolysaten für den Gebrauch als Kontrollmaterial für die Bestimmung glykosylierter Hämoglobine werden beschrieben. Diese Hämolysate wurden entsprechend den zur Reinigung von menschlichem Hämoglobin verwendeten Verfahren aus Blutproben Gesunder und von Diabetikern hergestellt. Zusätzlich wurde dialysiert, um Glucose aus den labilen Fraktionen zu entfernen, und mit Ethylenglykol verdünnt. Alle Hämoglobinfraktionen wurden in ihre CO-Derivate überführt, um ihre Stabilität zu erhöhen, und unter verschiedenen Bedingungen aufbewahrt.

Während zehnmonatiger Aufbewahrung bei −20 °C wurden keine signifikanten Änderungen der Konzentration glycosylierter Hämoglobine beobachtet.

Introduction

The measurement of glycated haemoglobins HbA_{1c} or HbA_1 is of great clinical importance for the long term monitoring of diabetic patients (1, 2). The clinical usefulness of this test is greatly enhanced when the so called „labile" fraction, i.e. the *Schiff* base adduct between haemoglobin and glucose, is dissociated by dialysis (3), treatment with a borate buffer (4), or with semicarbazide (5).

Unfortunely, no stable reference material is available. According to a recent report (6), control data are satisfactory only when the thiobarbituric acid test is used to measure calibration compounds (fructose or 5-hydroxymethylfurfural), or affinity chromatography is used with whole blood (7) to measure the glycated haemoglobins. Fructose or 5-hydroxymethylfurfural are easily available in the laboratory. Although whole blood can be used directly as control material (7), it can not be stored for a long time.

Some lyophilized standards are now commercially available (from Bio-Rad Laboratories and Boehringer Mannheim) but their stability after reconstitution is very limited.

In our previous reports on quality control in haemoglobinometry (8) and on the measurements of haemoglobins A_2 and F (9) we described the preparation of some ethylene glycol-stabilized haemolysates. These were shown to be stable for more than one year when stored at −20 °C.

In this paper, a similar technique is described for the preparation of haemolysates for use as control material for the assay of glycated haemoglobins. The following experimental steps were adopted:

a) removal of the labile fraction by incubation of the haemolysate with an appropriate borate buffer;
b) conversion of haemoglobin to the carbon monoxide derivative;
c) dilution of the solution with ethylene glycol and storage at −20 °C.

Materials and Methods

Preparation of the haemolysate

Haemolysates, containing normal and pathological HbA_1 concentrations, were prepared from blood samples from healthy subjects (from the blood center AVIS, Milan) and from a pool of washed red blood cells from diabetic patients, respectively. Sodium EDTA at 1 g/l final concentration was used as anticoagulant. No sample with abnormal values of HbA_2, HbF, HBsAg and/or abnormal haemoglobin bands on cellulose acetate electrophoresis at pH 8.4 or positive for HBsAg was included. All the purification work was performed in the cold room.

After plasma removal, the red blood cells were washed three times with a 9.0 g/l NaCl solution and then haemolysed by addition of CCl_4 and distilled water (0.4 volumes and 1.0 volumes, respectively, per volume of red blood cells). The haemoglobin solution was then separated after centrifugation at 3000 *g* for 30 min and dialysed against 50 mmol/l sodium tetraborate pH 8.9. After 4 changes of the buffer, the dialysing solution was replaced by a solution of 20 mmol/l K_2HPO_4, 50 mmol/l KCl and 5 mmol/l EDTA at pH 7.4. The dialysis was complete after 4 further changes of this buffer. The resulting haemoglobin solution was then concentrated with an Amicon apparatus (using PM 10 membranes) up to 230 g/l and flushed with carbon monoxide in sealed vials for about 5 min.

The haemoglobin was allowed to stand overnight under an atmosphere of CO, then centrifuged at 20000 g for 40 min to remove any precipitated material. Finally, it was diluted with ethylene glycol to give a total Hb concentration of about 150 g/l in 0.35 vol/vol ethylene glycol. Small aliquots (2 ml) of this material were then distributed in sterilized sealed vials, flushed again with carbon monoxide and stored at −20 °C.

HbA_1 and HbA_{1c} measurements

The HbA_1 and HbA_{1c} levels were determined by the commercial methods available from Bio-Rad Laboratories (Milan). All the chromatographic runs were performed in a thermostated chamber at 23 ± 0.2 °C.

Spectrophotometric analysis of haemoglobin solutions

The HbCO and MetHb contents of the haemoglobin solutions were determined by use of a multicomponent analysis programme on the spectrophotometer Sp 601 (C. Erba, Milan). The 10 mmol/l borate solution at pH 9.1 used for these analyses was prepared fresh each week.

The same instrument was used to obtain the haemoglobin spectra.

Isoelectric focusing

Isoelectric focusing (IEF) analysis of the samples was performed on a polyacrylamide gel (12 × 12 × 0.05 cm, T = 6%, C = 4%) containing 4% of LKB ampholines (pH 6−8). The run was performed at 5 W constant power and 800 V. Staining was done by the Comassie blue method as described by *Malik* (10).

Results

The HbA_1 and HbA_{1c} levels of the normal and of the diabetic haemolysates stored at −20 °C were analysed over a 10 month period and found to be very stable.

All the analyses were performed in duplicate on the same vials. The stability was statistically assessed by means of a linear regression analysis of analytical values (y, fraction) versus time of storage (x, in days) (11). The relevant parameters are shown in table 1. No significant differences were found between the overall mean values and the corresponding intercept values. The within run precision CV for the HbA_1 and HbA_{1c} determinations were 1.7% and 2.5% respectively. For the normal sample the between run CV were 3.3% for the HbA_1 determinations and 7.2% for the HbA_{1c}. For the diabetic samples they were 2.6% and 5.0%, respectively.

The IEF analysis (fig. 1) did not reveal significant differences among the samples, before and after about one year of storage at −20 °C.

Tab. 1. Regression analysis parameters calculated on the data of figure 1.

Haemolysate	Parameter	$y = HbA_1$	$y = HbA_{1c}$
Normal	slope	0.000	0.000
	SD	±0.001	±0.002
	intercept	7.2	4.9
	SD	±0.1	±0.1
	mean	7.1	4.9
	SD	±0.2	±0.3
Diabetic	slope	0.000	0.000
	SD	±0.001	±0.001
	intercept	10.3	7.4
	SD	±0.1	±0.1
	mean	10.2	7.4
	SD	±0.2	±0.3

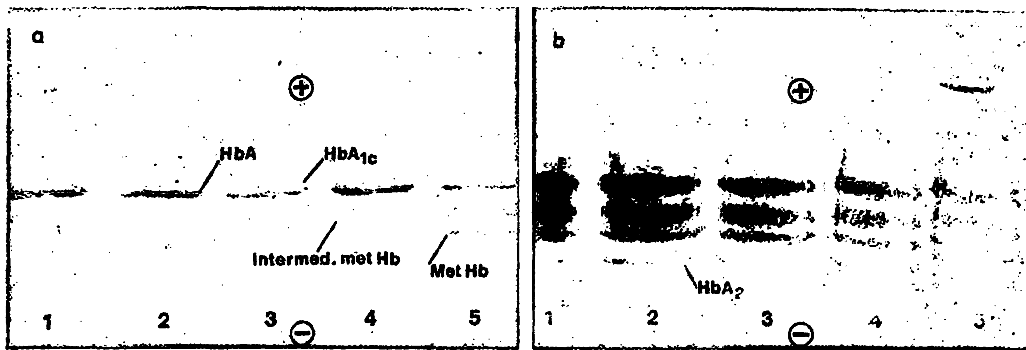

Fig. 1. Photographs of the IEF analysis on the haemolysates before and after one year of storage at −20°C.

Sample 1: freshly prepared normal haemolysate;
sample 2: the same as in 1, one year later;
sample 3: freshly prepared diabetic haemolysate;
sample 4: the same as in 3, one year later;
sample 5: oxyhaemoglobin solution partially oxidized (50% of the haem) with ferricyanide.

a) photograph taken soon after the IEF run.
b) the same slab as in A, after staining with Comassie blue.

By spectrophotometric analysis, the amount of MetHb in all samples was found to be neglegible; furthermore, no trace of haemoglobin aging components (such as HbA_3 and other, (13)) were detected.

After staining with Comassie blue the composition of the non-haemoglobin bands was also found to be unchanged in all the samples.

A third preparation from non diabetic blood was used to test the stability of HbA_1 and HbA_{1c} levels during 15 days storage at −80°C, +4°C, +25°C and +37°C.

The results are shown in figure 2.

The HbCO and MetHb concentrations of the haemolysates stored at −20°C were analysed on the same days that HbA_1 and HbA_{1c} measurements were made. The HbCO and MetHb levels were found to be stable during storage. Over 146 days of storage, the HbCO and MetHb levels were 91.3 ± 1.6% and 2.2 ± 0.2% (mean ± SD), respectively.

The HbCO and MetHb were also determined in the samples stored at −80°C, +4°C, +25°C and +37°C. No significative deviations from the initial values were reported during the period of storage.

To evaluate the possible use of this material in long term quality control programs for HbA_1 and HbA_{1c} measurements, we stored some haemolysate at −80°C. Under these conditions the solution freezes; we think, however, that the combined effect of the ethylene glycol and the carbon monoxide can prevent the haemoglobin from autoxidation. The effects of repeated cycles of freezing and thawing on the same material were therefore studied by measuring the absorption spectra in the region 500−650 nm.

No significative changes in the initial spectrum were detected even after 16 cycles.

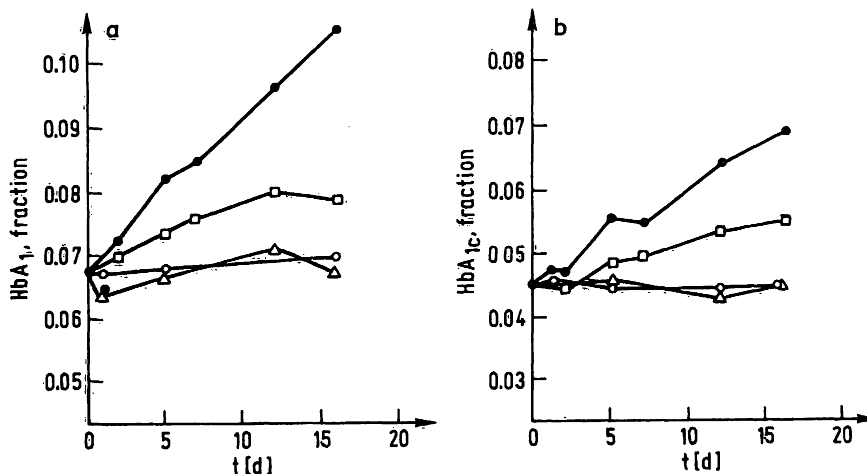

Fig. 2. HbA_1 (a) and HbA_{1c} (b) fractions in normal haemolysates stored at −80°C (△), +4°C (○), +25°C (□) and +37°C (●).

Discussion

It is known that protein solutions can be stabilized in several ways. Glycerol seems to have a powerful effect on the activity of several enzymes (12). In the case of haemoglobin, stabilization is improved by the use of carbon monoxide, preventing the generation of methaemoglobin, which is rapidly formed after exposure at relatively warm temperatures (8). Finally, the cold is probably a third protective agent and indeed our data demonstrate that under proper conditions of storage, the haemolysates can be maintained for long periods of time. The treatement with borate is particularly necessary to obtain haemolysates free from the „labile fractions".

A previous report by *Delgado & Daza* (14) described the preparation and the control of a lyophilized haemolysate which proved to be stable for 8 months after preparation. However no statistical analysis of the stability was presented. Further, the concentrations during the storage were not graphically illustrated.

We hope that the method described here will be helpful in the production of control materials for glycated haemoglobin assays, which is still, according to *Goldstein* (15), a very actual problem for the optimization of these analysis and for the comparison of values from clinic to clinic. As described above, we determined the two HbA_1 and HbA_{1c} concentrations by means of cation exchange chromatography on Bio-Rex 70 on Bio-Rad minicolumns. Although the real accuracy of the HbA_{1c} method could be questionable, nevertheless the two methods are among the most commonly used in clinical analysis. Of course, it is important to confirm our data using other techniques, such as HPLC and affinity chromatography. Work is now in progress in our laboratory to test a fresh stock of haemolysate by the thiobarbituric acid test and to evaluate the long term stability of haemolysates stored at −80 °C. The results of these experiments will be presented in a future communication.

The stabilized haemolysate herein described is now being tested by a group of the Community Bureau of Reference (Commission of the European Communities) as suitable material for an interlaboratories test. This test will be designed to evaluate analytical variability in the glycated haemoglobin measurement.

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