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Evaluation and improvement of a commercial method for routine chromatographic measurement of glycosylated hemoglobins

Valutazione e miglioramento di un metodo commerciale per la misura cromatografica di routine delle emoglobine glicosilate

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Summary: *Accurate quantitation of glycosylated hemoglobins may have a great clinical relevance for the control of diabetic patients. In the present paper a commercial technique for the detection of glycosylated hemoglobins is evaluated. Very high precision (within-run coefficient of variation 1.33%, compared to 3.03% referenced by Bio-Rad Laboratories) and accuracy (when this procedure was compared to a reference method, the correlation coefficient was 0.988) are achieved by the use of a temperature-controlled apparatus for the chromatographic columns. Some of the factors possibly causing variability in the assay of glycosylated hemoglobins are examined. Storage of blood for more than 8 hours at room temperature and 3 days at cold, and of hemolysates for more than 7 days at cold or frozen, must be avoided. Hemoglobin amounts in the hemolysate loaded on the column must not exceed 2.7 mg, corresponding to an hemoglobin concentration in blood of 18 g/100 ml, since for higher values overestimation of glycosylated hemoglobins may occur. When cells are required to be washed, care must be kept in not disturbing the cells after centrifugation, since their distribution in glycosylated hemoglobin content may not be randomized. Finally the mean value found in a population of 84 subjects with normal oral-glucose-tolerance test was 6.16 ± 0.63% (mean ± S D).*

Riassunto: *Nel presente lavoro è stata messa a punto una metodica commerciale per il dosaggio dell'emoglobina glicosilata nel sangue, parametro che ha assunto crescente importanza nel controllo dei pazienti diabetici. L'affinamento di questa metodica, basata su una cromatografia a scambio ionico su colonnine a perdere, unito all'uso di una speciale camera di termostatazione, ha permesso di raggiungere una notevole precisione ed accuratezza. In particolare è stato ottenuto un coefficiente di variazione pari a 1.33% nelle prove di precisione within-run (contro 3.03% riportato per il metodo originale) ed un coefficiente di correlazione pari a 0.988 fra la metodica qui descritta ed un metodo di riferimento. Sono stati inoltre esaminati alcuni fattori che possono essere causa di variabilità nel dosaggio dell'emoglobina glicosilata, quali la stabilità dei campioni di sangue e degli emolisati, la quantità di emoglobina caricata per colonna, la temperatura alla quale viene fatta la cromatografia e la non omogeneità dei campioni di sangue dopo centrifugazione. Infine sono riportati i risultati di uno studio svolto su una popolazione di 84 soggetti adulti con risposta normale al test di tolleranza al glucosio (v.n. 6.16 ± 0.63, media ± S D).*

Introduction

The glycosylation of human hemoglobin is a post transcriptional non-enzymatic process occuring throughout the life-span of the erythrocytes[1,2]. Glycosylated hemoglobins (HbA_{Ia-c}) are considered an integrated index of blood glucose levels over the period precedeng their measurement and are therefore of great clinical relevance for the long-term control of diabetic patients[3,4,5]. Recently, Dods et al[6] suggested a possible role of the assay of these minor fractions of human hemoglobin for the diagnosis of diabetes, as the less controversial and more practical parameter to be determined, since it is virtually insensitive to all those factors which can affect the results of oral-glucose-tolerance-test (OGTT). The importance of assaying glycosylated hemoglobins, unfortunately, is not always supported by an easily suitable and affidable technique. For example, drawbacks and uncertainties in the chromatographic methods, as lack of valid controls for evaluating the test and time-consuming procedures, may lead to rather controversial results. Moreover, simplification and feasibility of these methods by disposable ion exchange columns do not always correspond to more

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accurate and precise results. In the present paper one of these methods is throughly analyzed and the various factors which could affect precision and confidence are investigated.

Materials and Methods

Venous whole blood was obtained from normal and diabetic volunteers and collected in tubes containing EDTA as anticoagulant. Informed consent was given by all subjects. When not immediately processed the tubes were placed in an ice-bath at 0° C unless otherwise specified. Hemolysates were prepared by a five-times dilution of well stirred blood samples into the hemolyzing agent (0.33% polyoxyethylene ether) supplied by Bio-Rad Laboratories (2200 Wright Ave., Richmond, California 94804, USA) with precision devices such as microsyringes or micropettors. Disposable devices as pipets or automatic dispensers were avoided for lack of accuracy.

Disposable cationic exchange columns (0.8 by 2.3 cm) commercially available from Bio-Rad Laboratories for HbA_{Ia-c} assay were employed in all the experiments. Columns are packed with Bio-Rex 70 resin (200-400 mesh) and equilibrated to pH 6.7 with a phosphate-KCN buffer. The technique for separating fast glycosylated hemoglobins is a modification of the method described by Trivelli et al[3]. The major modifications consist in the preparation of the hemolysates from whole blood just before the chromatographic run without washing red blood cells and successive dialyzing, and in the collection of fast fractions in a fixed volume (10 ml) of buffer without elution of non-glycosylated hemoglobins. The percentage of HbA_{Ia-c} is calculated from the ratio of optical densities between fast and total hemoglobins. Spectrophotometric measurements were performed by a Spectracomp 601 (Carlo Erba Strumentazione, Milano, Italy) at 415 nm (bandwidth = 3.5 nm), with 1 cm pathlength glass cuvettes against a blank of buffer alone.

Chromatographic assays were performed at constant temperature with the working apparatus shown in Figure 1. A constant temperature circulator (a) is connected to a plexiglass compartment (b) (14.5 cm in diameter by 9 cm in height) in which up to ten columns may be fitted. This device lies on a rack (c) with tubes collecting the eluates. A fixed volume (10.0 ± 0.02 ml) of temperature equilibrated buffer is delivered by a precision burette (e), drawing out the buffer from a reservoir immersed in the same circulating bath (d). A thin silicone tubings connects the burette to the columns. Unless otherwise specified, the operating temperature was aluays $23.0° \pm 0.1°C$. The procedure proposed by Trivelli et al[3] and modified by Saibene et al[7] was considered the reference method for the measurement of HbA_{Ia-c}.

Total hemoglobin and methemoglobin concentrations in blood were detected by a Micro Blood Analyzer (Carlo Erba Strumentazione). For statistical analysis

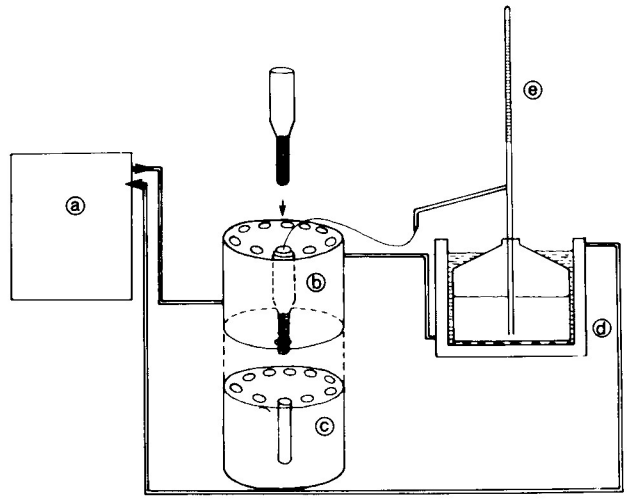

Fig. 1 - *Schematic representation of the apparatus employed in the present work: (a) Constant temperature circulator; (b) chromatographic columns compartment (patents pending); (c) tubes rack; (d) thermostated buffer reservoir; (e) precision microburette*

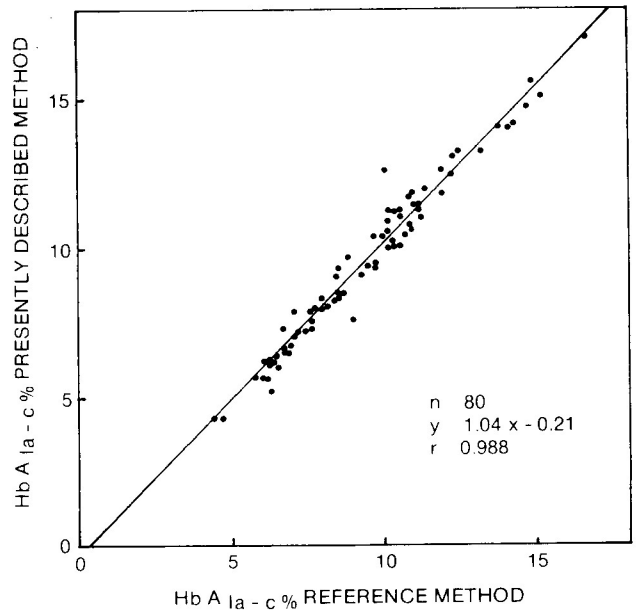

Fig. 2 - *Correlation between the method under investigation and the reference method*

of results, Student *t* test for paired and unpaired data, linear regression, correlation coefficient, and the coefficient of variation were applied as appropriate.

Results

Temperature

The effect of the operating temperature on the assay of HbA_{Ia-c} was investigated by proper use of the apparatus shown in Figure 1. No thermal losses were detected by a mercury bulb thermometer between the circulating bath and the resin contained in the column.

Four chromatographic runs of the same sample were

performed at a different temperature in the range 18°-33°C. Elution profiles were obtained collecting 18° fractions (about 0.55 ml each) for each run and reading the optical densities at 415 nm (Fig. 3). At the lowest temperature (18°C), fast fractions are not totally eluted, while at higher temperature (28° and 33°C) partial collection of slow fractions occurs with following over or underestimation, as summarized in Table I.

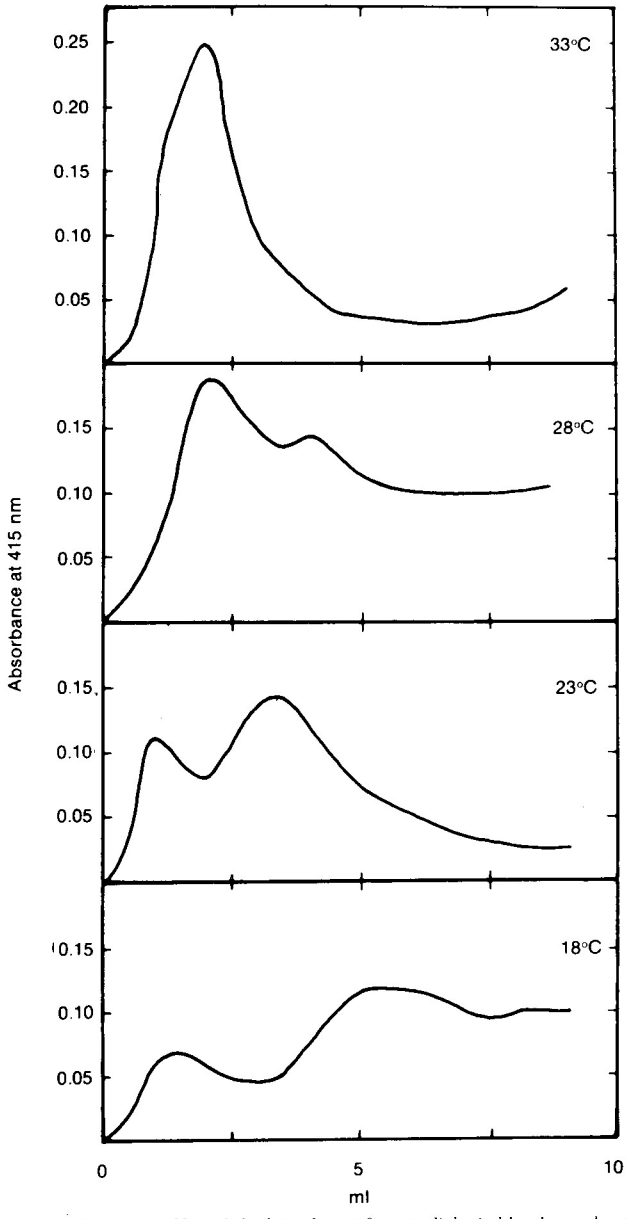

Fig. 3 - *Elution profiles of the hemolysate from a diabetic blood sample at various temperature. The profiles from a normal subject are essentially similar to those shown in figure*

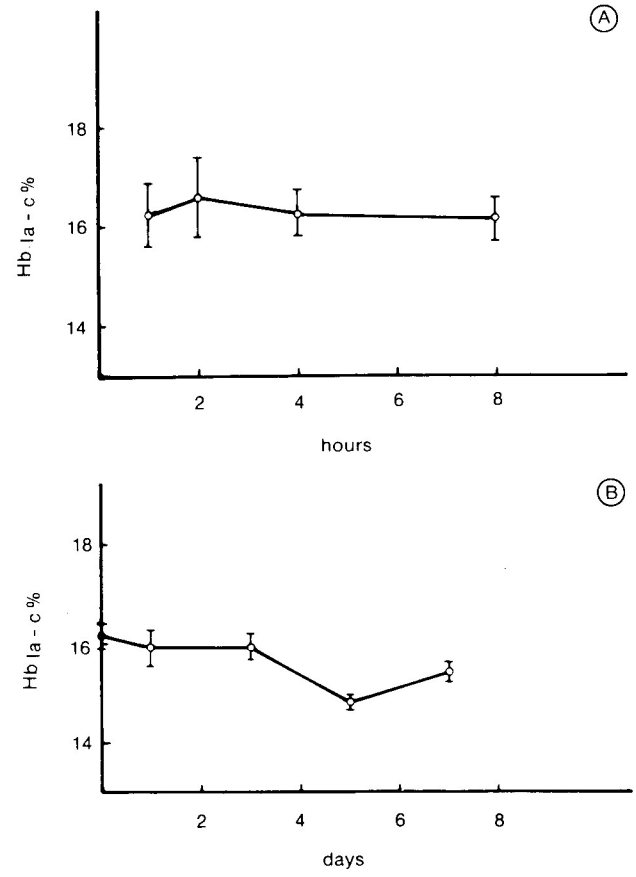

Fig. 4 - *Stability of HbAIa-c in a blood sample stored at 23°C (A) and at 4°C (B). The vertical bars represent the 95% confidence limits of the measurements*

Precision and accuracy

Sixty-one samples from normal and diabetic donors were run at 23°C in duplicate and occasionally triplicate in order to check the precision of the method, yielding a within-run coefficient of variation of 1.33%, compared to 3.03% referenced by Bio-Rad Laboratories.

Accuracy was evaluated by comparison with the reference chromatographic method running 80 samples in duplicate with both methods. Figure 2 shows the linear regression obtained from the measurements.

Storage of blood and hemolysates

The effect of storage of blood on HbA_{Ia-c} assay was investigated on whole blood from 3 diabetic patients and from 3 healthy volunteers. Figure 4 shows the most indicative results obtained when whole blood was stored in stoppered and sterilyzed tubes at room

Tab. I - *Effect of the elution temperature on the HbAIa-c value (mean ± S D)*

Subject	Temperature			
	18° C	23°C	28° C	33° C
Diabetic	15.63 ± 0.07	16.22 ± 0.20	18.49 ± 0.74	22.54 ± 0.16
Normal	6.67 ± 0.19	7.59 ± 0.14	8.98 ± 0.25	12.11 ± 0.32

temperature (23°C) and at cold (4°C). No hemolysis or abnormal values of methemoglobin were detected at the end of the storage time.
Hemolysates were stored in well stoppered plastic tubes both at 4°C and frozen down to -18°C. No appreciable alteration of the measured HbA_{Ia-c} was observed up to 7 days in both cases (Tab. II). Neither precipitates nor impurities were observed throughout the storage.

Tab. II - *Stability of HbA_{Ia-c} values in hemolysates. Most indicative results only are shown (mean ± S D)*

	Days	
	1	7
Refrigerated (4° C)	17.36 ± 0.20	16.96 ± 0.19
Frozen (−18° C)	15.12 ± 0.45	14.91 ± 0.27

Hemoglobin amounts loading the columns
Different amounts of hemoglobin (1 to 4 mg) in the same volume of hemolysate (0.1 ml) were loaded on the columns and processed as usual. Figure 5 shows that HbA_{Ia-c} percentage was constant in the range 1 to 2.7 mg, while abnormally high values were found for exceeding amounts of hemoglobin. The second x-axis of the figure represents the calculated corresponding values for total hemoglobin concentration in blood.

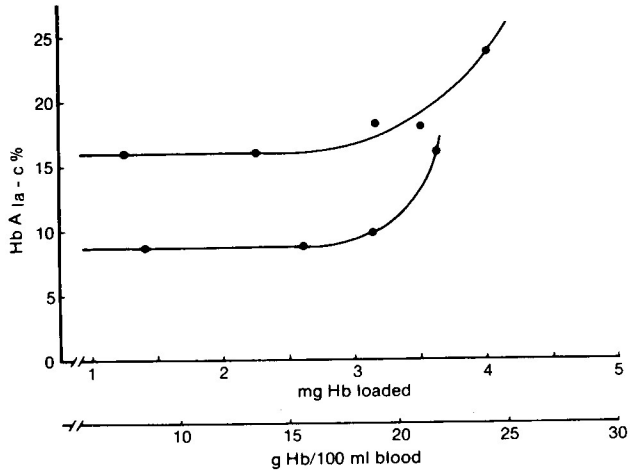

Fig. 5 - *Measured percentage of HbAIa-c as a function of the amount of hemoglobin loaded on the column*

Washing of red blood cells
HbA_{IA-c} assays were performed in 13 hemolysates obtained both from whole blood and from cells washed three times with isotonic saline (0.9% NaCl). A significant decrease of HbA_{Ia-c} values was evident after washings (14.25 ± 3.53 vs. 13.35 ± 3.66, $p < 0.02$). To evaluate the hypothesis that this decrease may be due to a non-uniform distribution of cells after the centrifugation, owing to their different sizes and to accidental aspiration of cells during washings, rather than to interferences of plasma, the following experiment was performed. A 10 ml blood sample was washed three times without disturbing the cells and finally centrifuged at 1,000 g for 10 min. Samples were then taken at the interface between the cells and the supernatant, in the middle and in the bottom of the cell suspension, and finally run separately. The resulting HbA_{Ia-c} percentages were 26.56 ± 0.06, 25.00 ± 0.05, 23.61 ± 0.04, respectively, while a separate run for a well stirred sample resulted in $25.37 + 0.06\%$. These results suggest that HbA_{Ia-c} rich cells lie on the top, while HbA_{Ia-c} poor cells lie in the bottom of the tube after centrifugation.

Normal value
HbA_{Ia-c} was measured in 84 adult subjects with normal oral-glucose-tolerance-test (50 g), according to Fajans and Conn criteria[8]. The results are in the legend of Figure 6. No significative difference was found according to sex or age.

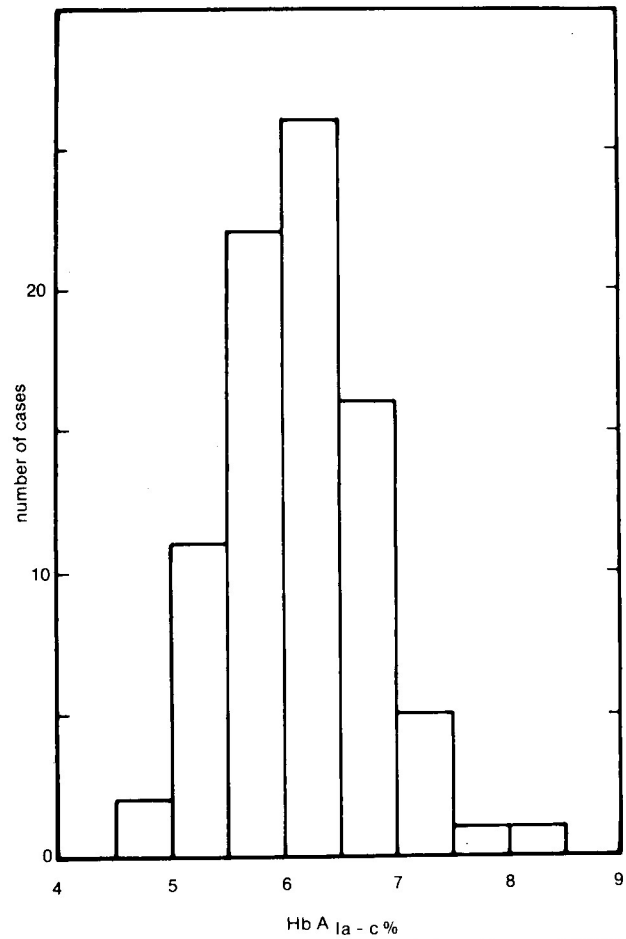

Fig. 6 - *Distribution of HbA_{Ia-c} in a healthy population with normal OGTT (n = 84 age, 11 to 54 years, 62 males and 22 females). Range 4.94-8.43%; median value 6.10; mean value 6.16 ± 0.63 (SD); 95% confidence limits between 7.42 and 4.90%*

Discussion

Several methods have been proposed for the detection of glycosylated hemoglobins. Cellulose acetate[9]

or starch gel electrophoretic separations give unsatisfactory resolution and have been no more applied for clinical purposes. Isoelectrofocusing employing a pH gradient modifier allows better resolution[10], but this technique is not suitable for routine use because of its complexity. Another approach to the problem of glycosylated hemoglobin detection is based on the measurement of 5-hydroxy-methyl-furfural (5-HMF) released by acid hydrolysis of glycoproteins[11]. This technique shows a satisfactory correlation with the chromatographic separation of HbA_{Ia-c}[7]. Recent observations[12] suggest however that human hemoglobin is also glycosylated by a glucose ketoamine linkage at other sites of the molecule, as the N-terminal positions of the alfa-chains and the epsilon-amino groups of several lysine residues, apart from the valines of the beta-chains. These groups may supply additional 5-HMF by acid hydrolysis, thus possibly giving overestimation of the values for HbA_{Ia-c}.

Chromatographic methods, first proposed by Schnek et al.[13], have probably been the most studied ones. They seem the most acceptable for clinical use since many improvements have been suggested in order to reduce bed volume and time consumption[14,15,16,17]. The simplification and commercial availability of many kits led however to a less satisfactory precision and accuracy in comparison to traditional procedures. Moreover, the importance of the operating temperature has been sometimes disregarded. Table I shows that great inaccuracy could derive from drifting of the operating temperature: eventual thermal variations in the laboratory may result in non-controlled fluctuations of the temperature in the column. This problem has been circumvented by other authors[6] introducing a correction factor for the operating temperature. An alternative experimental approach is presently suggested by keeping a constant operating temperature, achievable by the apparatus shown in Figure 1. Optimal operating temperature is 23°C, since the best resolution between fast and slow hemoglobins is obtained.

Apart from the dramatic effect of temperature on the assay of HbA_{Ia-c}, other factors in storing and handling samples may affect, although to a lesser extent, the affidability of the method and must therefore be taken into account. Variability in the results is evident when storing whole blood after sampling at room temperature. This inconvenient is almost totally prevented when keeping blood samples at cold (4°C). As for the hemoglobin concentration in the hemolysate, no detectable interference in the HbA_{Ia-c} assay derives from different amounts of hemoglobin eluted in the column in the range 1 to 2.7 mg, corresponding to a total blood hemoglobin concentration between 8 and 18 g/100 ml. An anemic condition, therefore, does not affect this assay, while hemoglobin values higher than 18 g/100 ml of blood may induce overestimation. In unusual clinical conditions, when this evenience may occur, blood must be preliminary diluted, in order to reduce the hemoglobin concentration of the hemolysate down to an acceptable value. The presently described method does not require preliminary cell washings; however, when employing other techniques requiring washings, care must be kept. In fact the concentration of HbA_{Ia-c} within the red cell is a function of the cell size or age[18], and differently sized cells stratify in well defined boundaries also for weak centrifugal forces, as 1,000 g for 10 min (see Results). Therefore accidental aspiration of some cells at the interface between the supernatant and the cell suspension during washings may change the distribution of the cell population, altering therefore the percentage of measured HbA_{Ia-c}, that is averaged on the whole population. These features may possibly explain the observed decrease of HbA_{Ia-c} throughout the washing cycle of the cells.

The normal value for HbA_{Ia-c} found by us in a healthy adult population is quite similar to analogous results published by other investigators[3,5,14,18].

This time-saving method (no more than one hour is required from blood sample receipt to the communication of the results) may be affidable both for routine analysis and for investigative purposes, provided adequate care in monitoring the operating temperature and in handling the samples is applied.

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