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QUANTITATIVE DETERMINATION OF HEMOGLOBIN A_1c BY THIN-LAYER ISOELECTRIC FOCUSING

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SUMMARY

A method for the quantitation of hemoglobin A_1c using isoelectric focusing is reported.

Hemolysates were prepared and stabilised with carbon monoxide and with potassium cyanide before the quantitation. The two preparations gave identical results. The potassium cyanide method is simple and adequate for routine purposes, but the cyanohemiglobin compound remains stable for one week only. The carbon monoxide method is more laborious, but the carboxyhemoglobin derivative remains stable for up to one year.

Quantitations of the separated fractions emerging after isoelectric focusing were made with spectrophotometry or with densitometry. No significant difference in the results could be shown. Reproducibility tests were improved by introducing transferrin as an internal standard.

The specificity of the method was checked by the in vitro addition of oral hypoglycemic drugs and of insulin.

INTRODUCTION

A hitherto unknown hemoglobin component in hemolysates prepared from the blood of diabetic subjects was first noted by Rahbar [1] and later identified as hemoglobin A_1c (HbA_1c). Koenig et al. [2] demonstrated that a high A_1c value was an indicator of poor diabetic regulation. Consequently, the development of adequate methods for the routine quantitative determination of HbA_1c is important.

Previously described methods for the determination of HbA_1c revealed different disadvantages. Trivelli et al. [3] developed a rather laborious ion-exchange chromatographic method with a low capacity and with no separation between the hemoglobin fractions F and A_1c. Modifications of the method of Trivelli et al. [3] have been described [4,5] but they all revealed lack of specificity, as no separation between HbA_1a, HbA_1b and HbA_1c, nor between the HbF and the HbA_1c fractions could be obtained.

This paper describes a rapid and specific method for quantitation of HbA_1c, adequate for clinical purposes in the monitoring of metabolic regulation in diabetic patients.

MATERIALS AND METHODS

Chemicals

The polyacrylamide gels (1 mm thick) specially designed for HbA_1c determinations (LKB Products, Bromma, Sweden) had a specific narrow-range pH gradient. The anolyte (0.05 *M* L-glutamic acid), the catholyte (0.1 *N* sodium hydroxide), potassium cyanide, tetrachloromethane and ammonium hydrogen carbonate were all of analytical grade (Merck, Darmstadt, G.F.R.). Human-transferrin was obtained from Behringwerke (Marburg-Lahn, G.F.R.). Chromatographically purified HbA_1c was obtained from Isolab. (Akron, OH, U.S.A.). Carbon monoxide was of specially purified grade. The staining solution consisted of 1 g Coomassie Brilliant Blue R-250 (Merck) dissolved in 1 l of an aqueous solution containing ethanol (25%) and acetic acid (8%). This solution should be freshly prepared every day. Fixing solution: 57.5 g trichloroacetic acid and 17.25 g sulphosalicylic acid were added to 500 ml distilled water. Destaining solution: 500 ml ethanol and 160 ml acetic acid were mixed and diluted to 2 l with distilled water. Preserving solution: 40 ml of glycerol were added to 400 ml of the destaining solution.

Equipment

The isoelectric focusing was carried out on a LKB Multiphor System 2117 equipped with a constant power supply LKB 2103. To maintain a constant temperature (15°C) a LKB multitemp 2209 was used. Sample application pieces were LKB Paratex (10 × 20 mm) and the electrode focusing strips were LKB 2118-106. The measurements were carried out on a spectrophotometer LKB 2074 and a soft laser scanner SL-504 from Biomedical Instruments (Chicago, IL, U.S.A.).

Preparation of hemolysates

A 2-ml volume of venous whole blood was collected in a heparin tube. A 160-µl volume of capillary blood was drawn into heparin coated microhematocrit tubes (Beckmann, Fullerton, CA, U.S.A.). Erythrocytes that had been washed three times with five volumes of saline were hemolysed with eight volumes of distilled water. The cell debris was extracted with half a volume of tetrachloromethane. After centrifugation for 30 min at 2000 *g* the upper layer was separated and equilibrated with carbon monoxide for 5 min at 37°C. This resulted in a concentration of hemoglobin equal to 2—2.5 mmol/l. The carboxyhemoglobin was stored at —70°C until analysis. Stored in this way the carboxyhemoglobin is stable for one year [6].

The cyanohemiglobin method was as described for the carboxyhemoglobin method except that 0.03 *M* potassium cyanide was used to hemolyse the erythrocytes.

Preparation of hemolysates, densitometric method

The preparation of the hemolysates was as described under the spectrophotometric method except for the addition of transferrin as the internal standard (1.5 mg) for venous, and (0.4 mg) for capillary blood samples. Furthermore the concentration of total hemoglobin in the hemolysates was diminished 30 times by dilution with distilled water. This resulted in a hemoglobin concentration equal to 60—100 μmol/l before application on the gel slab.

Isoelectric focusing

The gel was placed on a glass cooling plate in an electrophoresis box and kept at 15°C during the separation procedure.

The gel was pre-run at 20 W for 30 min. Next, 30 sample application pieces were placed at a distance of 5 mm from the cathodic electrode focusing strip. Each sample application piece was covered with 15 μl of hemolysate and the gel was supplied with a constant effect of 20 W for 2 h. The hemolysate had been quantitatively removed from the sample application pieces within the first 30 min of electrophoresis. At that time the application pieces were removed to avoid tailing phenomena. To ensure maximum separation the gel was supplied with an effect of 30 W for the last 10 min.

Evaluation of hemoglobin A_1c concentration

Spectrophotometric method. The hemoglobin A_1c bands were cut off and transferred to plastic tubes containing 1 ml of a 1:1 mixture of 0.05 *M* ammonium hydrogen carbonate and 0.015 *M* potassium cyanide buffered to pH 8.4, and eluted in a rotary mixer (20 rpm) for 4 h. The carboxyhemoglobin samples were eluted in ammonium hydrogen carbonate only.

The total concentration of hemoglobin (C_{Hb}) in the hemolysate was determined by dilution of 15 μl of the hemolysate in 10 ml of ammonium hydrogen carbonate.

The extinctions of carboxyhemoglobin and cyanohemiglobin were measured spectrophotometrically at 417 nm. The concentrations of the components were determined from the molar extinction coefficients (see below).

$$\frac{\text{Ext. (total, COHb)} \times \text{dilution (5341)} \times 10^3}{201{,}000} = C_{COHb}$$

The HbA_1c concentration was calculated in percent of total hemoglobin (measured as COHb):

$$\frac{\text{Ext. } (HbA_1c) \times 100}{\text{Ext. (total, COHb)} \times \text{dilution (10)}} = P_{HbA_1c}$$

Consequently, the concentration of HbA_1c expressed in mmol/l may be calculated from the formula:

$$C_{HbA_1c} = P_{HbA_1c} \times C_{COHb}$$

To improve the spectrophotometric measurements a carbon monoxide stabilised hemolysate with known HbA_1c concentration was analysed every day. The hemolysate was kept at −70°C.

Densitometric method. On completion of the isoelectric focusing procedure the gel was stained according to the method described by Jeppson et al. [6]. The HbA_1c concentrations were read from standard curves made from densitometer traces of samples containing varying but known concentrations of HbA_1c as evaluated by the spectrophotometric method (cf. Fig. 3).

RESULTS

Measurements of dilutions (1:70 to 1:63,000) were carried out for venous as well as for capillary blood samples, using both the cyanohemiglobin as well as the carboxyhemoglobin methods (Table I). Proportionality between extinction and concentration was obtained for hemoglobin concentrations below 2.7 μmol/l. The extinction coefficients of the two hemoglobin derivatives used in the calculations of the HbA_1c are 201,000 mol^{-1} cm^{-1} l and 222,000 mol^{-1} cm^{-1} l for carboxyhemoglobin and cyanohemiglobin, respectively. The values were calculated by using the formula: $E \times \text{dilution} = \epsilon \times c \times 1$ where E is the measured extinction value, c is the hemoglobin concentration and ϵ the extinction coefficient. The mean (n=10) of the extinction values measured on hemolysates diluted 5341 times was used as the E value. These measurements were done at room temperature on carbon monoxide stabilised as well as potassium cyanide stabilised hemolysates. The concentration of total hemoglobin was measured spectrophotometrically (n=10) and the mean used as the c value (Van Kampen's modification of Drabkin's method [7]). The corresponding ϵ values were calculated from the formula.

TABLE I

EVALUATION OF THE RANGE FOR TOTAL HEMOGLOBIN IN THE HEMOLYSATES GIVING DIRECT PROPORTIONALITY BETWEEN THE CONCENTRATIONS AND THE OBTAINED EXTINCTION VALUES

Measurements done using carbon monoxide stabilised hemolysates.

Dilution of hemolysate	Extinction value (cm^{-1})	Calculated conc. of hemoglobin in the hemolysates (μmol/l)*
70	2.545	12.661
700	1.278	6.358
1400	1.270	6.318
2100	0.917	4.562
2800	0.713	3.547
3500	0.548	2.726
7000	0.260	1.297
14,000	0.129	0.642
21,000	0.081	0.403
28,000	0.061	0.303
49,000	0.038	0.189
63,000	0.022	0.109

*$C_{Hb} = \frac{\text{Ext.} \times 10^6}{201{,}000}$

To determine the optimal concentration of hemoglobin giving the best separation, samples containing different concentrations of total hemoglobin were isoelectrically focused (Fig. 1). Day-to-day variations revealed the smallest deviations using hemolysates containing total hemoglobin in the concentration range 2.0—2.5 mmol/l. The coefficient of variation (C.V.) was never above 4% measuring the same hemolysates five times within nine days. The carboxyhemoglobin and the cyanohemiglobin methods were shown to give identical results. Nine samples were analysed using both methods. Mean ± S.D. for the two methods were 0.78 ± 0.02 and 0.78 ± 0.03 mmol/l, respectively.

Comparison between the spectrophotometric and a new densitometric quantitation was carried out. A condition for obtaining reliable results is that the range for the total hemoglobin concentration in the hemolysates is of an order giving direct proportionality between the transmission of the monochromatic laser light through the HbA_1c band and the concentration of the hemoglobin fraction. Measurements were carried out using different dilutions of the same hemolysate. The peak area of the HbA_1c peak in the densitometer trace (Fig. 2) was compared to the corresponding concentration (Table II). As demonstrated a total hemoglobin concentration up to 248 μmol/l revealed direct proportionality between the HbA_1c concentration present and the corresponding peak area.

The calibration of the densitometer was carried out by analysing the HbA_1c concentration in hemolysates from non-diabetic as well as from diabetic subjects. Each concentration was spectrophotometrically determined (carbon monoxide method) as the mean of five determinations. Hemolysates from the

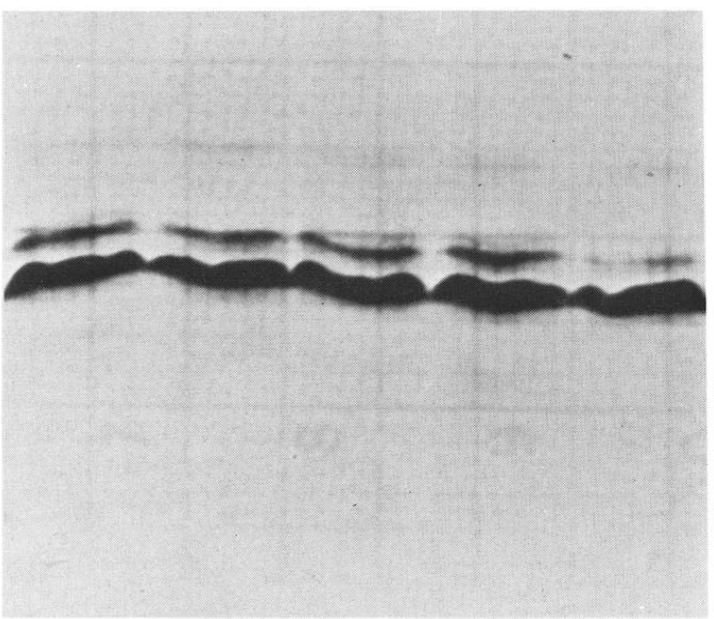

Fig. 1. Typical hemoglobin isoelectric focusing patterns from different patients. The HbA_1c fraction (upper band) is demonstrated to be clearly separated from the HbA_1 fraction (lower band). The four left-hand tracks are from diabetics, and the right-hand track is a smaller HbA_1c fraction from a non-diabetic.

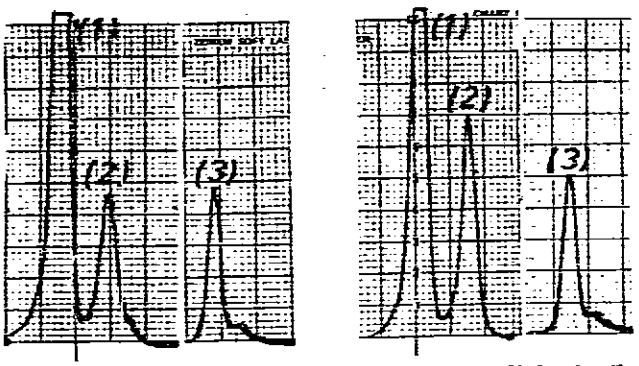

Fig. 2. Densitometer traces from a non-diabetic (left) and from a diabetic subject (right). The HbA_1c peak (2) is substantially higher in the right chromatogram correlated to the transferrin peak (3). The HbA_1 fraction is represented by peak (1). The *y*-axis gives the relative intensity, and the *x*-axis the paper speed.

TABLE II

EVALUATION OF THE RANGE FOR TOTAL HEMOGLOBIN CONCENTRATIONS IN THE HEMOLYSATES GIVING DIRECT PROPORTIONALITY BETWEEN THE CONTENT OF HbA_1c AND THE OBTAINED HbA_1c PEAK AREAS ACHIEVED BY THE DENSITOMETRIC QUANTITATION

Concentration of total hemoglobin in the hemolysate (carbon monoxide stabilised) (μmol/l)	Peak area (arbitrary units) by densitometric measurements of HbA_1c
62	9.0
93	14.0
124	23.0
155	26.0
186	30.0
248	40.0
310	68.5
434	82.0
620	95.0

same subjects were then densitometrically quantitated using the peak area of HbA_1c. The method was improved substantially by adding the same amount of transferrin as an internal standard to each application piece before the isoelectric focusing procedure. The peak area of HbA_1c and the ratio (R) between the peak areas of HbA_1c and transferrin were used as a measure for the HbA_1c concentration (Fig. 3).

The comparability of the spectrophotometric and the densitometric method was investigated by analysing ten different samples using both methods. No significant difference between the methods was found using the paired *t*-test.

The reproducibility of the densitometric method was carried out by analysing the same sample ten times within the same day (mean ± S.D. = 0.73 ± 0.03 mmol/l).

The proof of the identity of the HbA_1c band on the gel was carried out by comparing the isoelectric focusing parameters of the chromatographically purified HbA_1c.

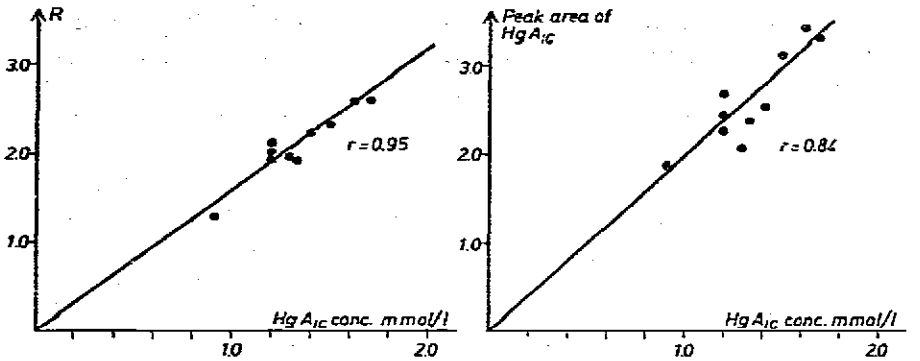

Fig. 3. Calibration curves constructed on the basis of known HbA_1c concentrations plotted against peak area of HbA_1c (right) and the ratio of peak areas of HbA_1c and the internal standard (left).

The specificity of the methods was examined by measuring the HbA_1c concentration in hemolysates with and without the addition of drugs commonly used by diabetics. Two drugs, metformine and tolbutamide, were added in amounts giving a concentration equal to 1 g/l hemolysate. Regular insulin (monocomponent) was added in amounts of 1200 IU/l. In no cases, either using spectrophotometry or densitometry, did the addition of these hypoglycemic drugs influence the HbA_1c concentrations.

DISCUSSION

The absorption maxima of carboxyhemoglobin and cyanohemiglobin have both been reported to be 421 nm [8]. However, our experiments using distilled water for dilution revealed an absorption maximum at 417 nm (25°C). To ensure that the maximum absorption wavelengths of the compounds were independent of the concentrations, spectral analyses were carried out on carboxyhemoglobin and cyanohemiglobin solutions in different concentrations. No influence on the absorption maximum of the concentration was found within the usual range of concentrations (0.6—2.7 μmol/l).

Our experiments revealed that whole blood when kept in a refrigerator (4—6°C) is stable for five days in the absence of stabilising agent. Hemolysates stabilised with potassium cyanide are stable for a period of up to nine days. This could be due to the formation of a hemoglobin A_3 fraction giving a reduction of the HbA_1c value [6]. Spicer et al. [9] found that after the addition of glycerol to the cyanohemiglobin it was possible to store the derivative at —12°C for a longer period of time. Our investigations did not confirm this finding. Deep freezing of the hemolysate should be avoided under all circumstances, due to flocculations in the hemolysate.

Experiments indicated that spectrophotometric determinations gave identical results independent of the stabilising agent (carbon monoxide or potassium cyanide). Furthermore the concentration of HbA_1c is shown to be equal in capillary as well as in venous blood samples. Identical results are obtained for total hemoglobin concentrations in the hemolysates with concentrations between 2 and 2.5 mmol/l. Reproducibility tests display very small deviations

with a coefficient of variation not greater than 4%. Furthermore the day-to-day variations as measured five times within nine days revealed only small fluctuations sufficient to claim a steady level in the clinic.

A very important parameter when using isoelectric focusing is the application of an optimal amount of total hemoglobin on the application pieces. In order to quantitate the concentration of HbA_1c by spectrophotometry relatively large amounts of hemoglobin (30—40 nmol) have to be isoelectrically focused. However, too much hemoglobin causes poor separation. Therefore, optimal conditions must be determined experimentally. Table I shows direct proportionality between the extinction values obtained and the degree of dilution for concentrations of total hemoglobin in the hemolysates ranging from 0.6 to 2.7 μmol/l. This concentration range ensures an adequate HbA_1c amount on the separated gel band. Isoelectric focusing followed by densitometric quantitation of HbA_1c requires an amount of 1—2 nmol total hemoglobin to ensure sufficient sensitivity. As demonstrated in Table II no amounts of total hemoglobin larger than 2 nmol should be applied (15 μl of 155 μmol/l) if proportionality between concentrations and the HbA_1c peak areas is to be ensured. Fig. 2 illustrates the densitometer trace of a HbA_1c determination. The separation between HbA_1 and HbA_1c is not complete, giving an additional area to the HbA_1c peak. However, this additional area has no significance and as demonstrated in Fig. 3 a straight calibration line is achieved when plotting the ratio of the peak areas against the concentration of HbA_1c. In conclusion, spectrophotometric and densitometric quantitations of HbA_1c have been demonstrated to give equal sensitivity and reproducibility. This means that results obtained by different groups using either one of the two methods can be immediately compared. This is of the utmost importance for utilization of these measurements routinely.

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