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A Rapid Microchromatographic Method for Determination of Hemoglobin A_I

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ABSTRACT

Hemoglobin A_I (HbA_I) is a minor hemoglobin fraction, which is continuously formed by a glycosylation reaction throughout the life-span of the erythrocyte. The concentration of HbA_I reflects the average blood glucose level during the preceding 1 - 2 months and HbA_I-determinations have been found to be of value in long-term diabetes control. A simple and reliable microchromatographic method for HbA_I-determinations, suitable for routine clinical analyses, is described. In a group of diabetic patients (n = 126) the mean (± S.D.) HbA_I--value was 9.6 ± 1.8 %, in the reference group (n = 33) the corresponding value was 6.0 ± 0.8 %. A commercial kit (Bio-Rad) based on the same principle was compared with our method. It was of equal accuracy and gave comparable results.

INTRODUCTION

Hemoglobin A_I (HbA_I) is a heterogenous fraction composed of glycosylated hemoglobins and normally comprises about 6 % of the hemoglobin of the erythrocyte (1). HbA_I can be separated in several subfractions, HbA_{Ia}, HbA_{Ib} and HbA_{Ic}, of which HbA_{Ic} is preponderant. HbA_{Ic} is formed by a slow and essentially irreversible postsynthetic glycosylation reaction, taking place throughout the life span of the erythrocyte (2). The finding that the concentration of HbA_{Ic} reflects the average blood glucose level over a considerable time span (3, 4), initiated several studies on the possible use of glycosylated hemoglobins as an index for the long-term glucose regulation in diabetes. These studies were, however, hampered by the lack of rapid and simple methods for determining glycosylated hemoglobins. The original method of Trivelli *et al.* (1) based on chromatography on large columns of Bio-Rex 70, was tedious and therefore several micro-modifications of this method were developed (4, 5, 6, 7, 8). Since these micro-techniques are sensitive to minor changes in the experimental conditions (9), it is not known to what extent they are suitable for routine clinical use. In this paper we present a technique, which is simple and reliable and seems suitable for clinical work. A commercial kit based on the same prin-

ciple has been compared with this method and was found to give comparable results. Preliminary reports have been published earlier (10, 11).

MATERIAL AND METHODS

HbA_I-assay, own method

Erythrocytes from heparinized blood were washed twice with 0.9 % NaCl and hemolysed with one volume of distilled water and 0.2 volume of CCl_4. Centrifugations were performed for five minutes at room temperature at 3,000 rpm. Each hemolysate was then subjected to chromatography in duplicate. If the hemolysates were not analyzed at once, they were frozen at -80°C. Storage of the hemolysates at this temperature for several months did not affect the amount of HbA_I.

Bio-Rex (200 - 400 Mesh) was obtained from Bio-Rad Laboratories, Richmond, California. A suitable batch of Bio-Rex was prepared from 50 g of resin which was suspended in water, decanted twice and pH was adjusted to 6.3 with 1.5 M H_3PO_4. It was then rinsed on a filter first with 100 ml of a buffer containing 23 g $NaH_2PO_4 \cdot H_2O$ and 5.9 g Na_2HPO_4 in one liter of water, and then with 750 ml of the same buffer diluted five times. The latter buffer corresponds to Developer No 6 of Schnek and Schroeder (12) but omitting the KCN. The resin was then suspended in this buffer and poured into small polypropylene columns (0.7 x 4 cm with a 10 ml reservoir, Bio-Rad laboratories) to a resin volume of 1 ml. Before chromatography, each column was equilibrated with 4 ml of Developer No 6. Great care was taken to keep the temperature as constant as possible (22 $\pm$ 1°C) during chromatography, since higher HbA_I-values were obtained with increasing temperature (13).

Hemolysate corresponding to 2.0 -2.5 mg of hemoglobin was applied to each column which was eluted with Developer No 6. HbA_I was then clearly visible as a red band moving down the column, while the main part of the hemoglobin was retarded. The first 10 - 15 ml of effluent was discarded. HbA_I was generally eluted after 30 min and collected in one 10 ml fraction. The extinction at 415 nm was determined in a Hitachi model 101 spectrophotometer equipped with an Optilab multilog 801. From the mM extinction coefficient for oxyhemoglobin at 415 nm (131), the amount of eluted HbA_I was determined (14). HbA_I was expressed as percent of total hemoglobin applied to the column which was determined according to Drabkin (15). An aliquot of frozen hemolysate (-80°C) containing about 10 % HbA_I was always run in parallel with each lot of 10 unknown samples.

The eluted hemoglobin fractions were analysed by isoelectric focusing, using an LKB-apparatus and ready-made ampholine PAG-plates for thin layer electrofocusing (pH range 5.5 - 8.5).

HbA_I-assay, Bio-Rad method

A commercial kit for the determination of HbA_I was obtained from Bio-Rad Laboratories, Richmond, California. It contained prepacked columns, elution buffer, hemolysis reagent and HbA_I-standards. The assay was performed as described in the manual (16).

Other methods

Blood and urinary glucose were determined by the glucose-oxidase technique according to Hjelm and De Verdier (17).

Subjects

The reference group consisted of 33 apparently healthy men and women in the age of 20 to 40 years. In the subjects with the highest HbA_I-values fasting blood glucose was measured. In no individual did the level exceed the upper normal limit (5.7 mmol/l). A diabetic group of 126 patients who regularly attended the out-patient service for diabetic care was studied. Seventy-five of these were receiving insulin; the other 51 patients were treated with sulfonylurea drugs or in five cases with diet alone. Blood samples for determination of HbA_I and glucose were obtained in the morning before breakfast and before the regular dose of insulin or tablets. Each patient brought a 24 h urinary specimen which was analysed for glucose.

RESULTS

Capacity and accuracy of the method

With this technique, 30 - 40 samples could be analyzed daily by one technician. The intra- and inter-assay coefficient of variation was 3 % and 9 %, respectively. By isoelectric focusing it could be demonstrated that the isolated HbA_I-fraction contained one main component migrating as HbA_{Ic} and some minor components probably representing HbA_{Ia} and HbA_{Ib} (18).

HbA_I-values in normal diabetic subjects

The mean ($\pm$ S.D.) value of HbA_I in the reference group was 6.0 $\pm$ 0.8 %. There was no significant difference between male and female subjects. The mean ($\pm$ S.D.) HbA_I-value in the whole group of diabetic patients was 9.6 $\pm$ 1.8 %, which was significantly higher than in the reference group (Table I). There was no significant difference in HbA_I-values between insulin-treated patients and those treated with sulfonylurea drugs or diet alone (Table I).

Table I. Determination of HbA_I.

No.	Subjects	HbA_I % Mean ± S.D.
33	Healthy subjects	6.0 ± 0.8
126	All diabetics	9.6 ± 1.8
75	Diabetics, insulin treatment	9.8 ± 1.7
51	Diabetics, sulfonylurea/diet treatment	9.4 ± 1.8

Comparison with the Bio-Rad method

A similar microchromatographic technique (Bio-Rad) available as a commercial kit was compared with our method. When the two methods were used simultaneously in the reference group and in 33 of the diabetic patients, the Bio-Rad method yielded somewhat higher values (Table II). The correlation between the methods (Fig. 1) when applied to the diabetic patients was satisfactory (r = 0.94). The accuracy of the kit-technique was similar to ours with an intra- and inter-assay coefficient of variation of 3 % and 6 %, respectively.

Table II. Comparison between the two methods.

No.	Group of subjects	HbA_I (mean ± S.D.) Own method	Bio-Rad method
33	Reference group	6.0 ± 0.8	6.6 ± 0.4
33	Diabetic group	9.4 ± 2.4	9.7 ± 2.8

DISCUSSION

In this study, a micromodification of the chromatographic procedure of Trivelli *et al.* was used for determining HbA_I. Similar methods using simplified techniques have recently been described (4, 5, 6, 7) and are also commercially available (16, 19). With these methods, HbA_I is eluted as one fraction and it is generally not possible to determine HbA_{Ic} specifically as with the original method of Trivelli. This is probably of minor importance in clinical work since changes in HbA_{Ic} are known to be paralleled by corresponding changes in the total HbA_I-fraction (20, 21).

Microchromatographic procedures for determining HbA_I are sensitive to minor changes in the experimental conditions (9). Many reportes on new micro-column techniques do not contain adequate quality control data and thus the suitability of these techniques for routine clinical work cannot be properly assessed. The chromatographic step in our method has been standardized with respect to elution time, temperature and amount of hemoglobin applied to the columns. Further as the preparation of the resin is critical, the washing procedure must be descri-

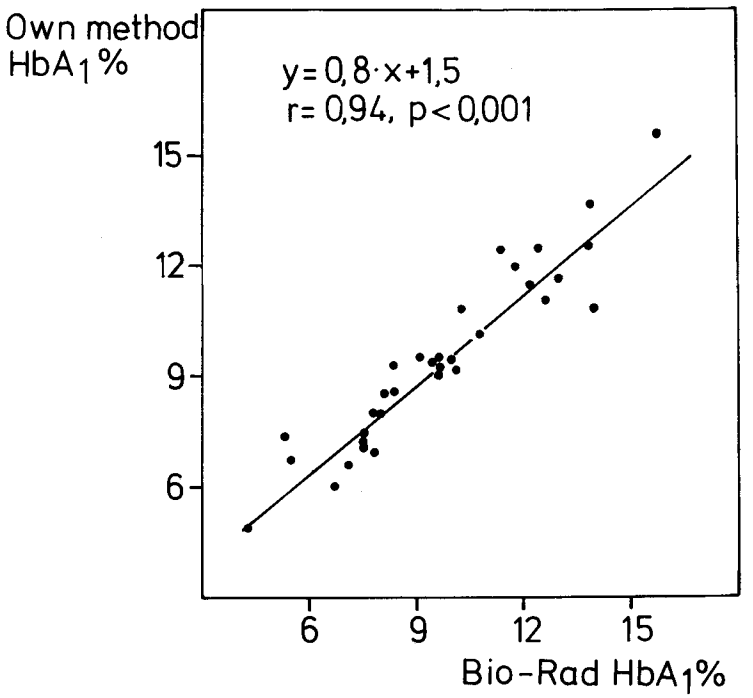

Fig. 1. Correlation between HbA_I-values obtained with the present method and with that of Bio-Rad in 33 diabetics.

bed in detail. With these precautions, it was possible to obtain a HbA_I-assay of satisfactory accuracy. The chromatographic step was rapid, which allowed the technician to handle 30 - 40 samples a day. The speed could be further increased by using an automatic spectrophotometer for the E_{415}-readings. Only 50 µl blood is needed for each analysis and therefore capillary blood samples can be used which is of particular importance when performing the analysis in children.

The HbA_I-values obtained with our method were in accordance with the data from previous reports (1, 4, 22, 23). It should, however, be pointed out that each laboratory performing HbA_I-analyses must set up its own reference values and "cut off" points for various degrees of diabetic control. It is also important to define the reference group with respect to age, since HbA_I-values have been shown to increase with age (23, 24). On the other hand, no significant difference was found between male and female subjects

The commercial kit from Bio-Rad was found to give values in good agreement with those obtained with our method. The use of prepacked columns was convenient since the preparation of the resin has to be carefully standardized in order to obtain batches with the same properties. The Bio-Rad method was a little quicker as the hemolysate was prepared from whole blood and not from separated erythrocytes as in our method. This procedure might, however, introduce an error if

the blood serum after Bio-Rex chromatography contains components which influence the E_{415}-values. This has, for instance, been shown to be the case with highly lipemic samples (25).

Other methods such as isoelectric focusing (18, 26) and high pressure chromatography (20, 27) are also available for HbA_I-determinations, but only a few laboratories and hospitals have the necessary equipment for these types of analyses. A colorimetric method based on the reaction between thiobarbituric acid and glycosylated hemoglobin residues, has been elaborated but does not have the desired accuracy (28). Likewise the principle for a radioimmunoassay has been described (29).

Although several problems concerning hemoglobin A_I remain to be solved, it now seems established that HbA_I-determinations will be of considerable value for assessment of the degree of diabetes control (24, 30, 31). No doubt, much effort will be made to improve present methods for HbA_I-determinations and to develop new techniques. Of the methods at present available, microchromatographic techniques being both simple and reliable seem to be preferable.

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