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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC SEPARATION AND QUANTITATION OF GLYCOSYLATED HEMOGLOBIN A_2 AS AN ALTERNATE INDEX OF GLYCEMIC CONTROL

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SUMMARY

By a combination of DEAE-cellulose chromatography and cation-exchange high-performance liquid chromatography glycosylated components of hemoglobin (Hb) A_2 were separated and quantitated from persons with diabetes and some common hemoglobinopathies. Hb A_{2Ic} values correlated well with total glycosylated Hb levels assayed by affinity chromatography, and Hb A_{Ic}, Hb S_{Ic} and Hb C_{Ic} levels, determined by high-performance liquid chromatography. The results indicate that Hb A_{2Ic} may serve as an alternate index of glycemic control.

INTRODUCTION

In addition to the major hemoglobin (Hb A) and a trace amount of fetal hemoglobin (Hb F), red cells from normal adults contain about 2.5% of Hb A_2 [1]. In patients with β-thalassemia the Hb A_2 concentration can be markedly elevated [2]. Several glycosylated minor components of Hb A, namely Hb A_{Ia1}, Hb A_{Ia2}, Hb A_{Ib}, Hb A_{Ic} and Hb A_{Id} have been identified [3–5]. Hb A_{Ic}, which constitutes the major portion of the minor hemoglobins, is increased two-to-three fold in diabetic patients [3, 5]. Hb A_{Ic} has a hexose moiety attached to the NH_2-terminal groups of the β-chains, whilst Hb A_{Ia1} and Hb A_{Ia2} seem to be the adducts of Hb A formed with phosphorylated glycolytic intermediates [3, 4]. Hb A_{Id} is formed predominantly by glycosylation of the α-chain NH_2-terminus and partly by disulfide interchange reaction of the β-chain cysteines with oxidized glutathione [3, 5]. Other glycosylated hemoglobins which are not normally separated by cation-exchange chromato-

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graphy and formed by glycosylation of ϵ-NH_2 groups lysines are also present in the red cell [3].

Because of the similarity between the β-chain of Hb A and the δ-chain of Hb A_2, one can expect a portion of Hb A_2 present in the glycosylated form. However, the quantity of the glycosylated Hb A_2 components in red cell hemolysates will be too small to be detected. Therefore the separation of the Hb A_2 minor components may require preliminary isolation of Hb A_2. Tegos and Beutler [6] have purified Hb A_2 by DE-52 chromatography and the isolated Hb A_2 was then subjected to isoelectric focusing which separated Hb A_{2Ia}, Hb A_{2Ib} and Hb A_{2Ic}. In this paper, we describe Bio-Rex 70 chromatographic and high-performance liquid chromatographic (HPLC) separation of the various components of Hb A_2 in normals, diabetics and in patients with various hemoglobinopathies. We also examined the possibility of using Hb A_{2Ic} as a tool for the assessment of glycemic control.

EXPERIMENTAL

Blood samples

After obtaining informed consent, blood samples were obtained in EDTA-containing Vacutainer tubes. Diabetic blood samples were obtained from the Diabetic Clinic and blood samples containing abnormal hemoglobins from the Sickle Cell Center of the Medical College. Red cell hemolysates were prepared from saline-washed red blood cells as described elsewhere [1].

DEAE cellulose chromatography for isolation of Hb A_2

For isolation of Hb A_2 for subsequent Bio-Rex 70 chromatographic separation of the minor components of Hb A_2, 1000 mg of red cell hemolysate were applied to a 25 × 2.5 cm column of DE-52 and developed with glycine—potassium cyanide—sodium chloride developers as described before [7]. For isolation of smaller quantities of Hb A_2 for subsequent HPLC separation of its minor components, 100 mg hemolysate were applied to a 25 × 1 cm column of DE-52.

Bio-Rex 70 chromatographic separation of the minor Hb A_2 components

Isolated Hb A_2 (50—100 mg) from blood samples of a normal adult and a diabetic patient was chromatographed on 25 × 2 cm columns of Bio-Rex 70 and developed by applying a linear sodium phosphate gradient with developers 6, 9 and 10 as described before [8, 9]. A flow-rate of 19–20 ml/h was maintained and 6.3—6.6 ml fractions were collected. Hb A_{2a+b}, Hb A_{2Ic} and Hb A_{2o} fractions separated in this manner were pooled and concentrated for subsequent chemical analyses.

Thiobarbituric acid assay for ketoamine content

For analysis of protein-bound ketoamine in the Hb components, a modified colorimetric assay by Standefer and Eaton [10] was followed.

Determination of phosphate

A 5-mg amount of hemoglobin was used to determine the protein-bound phosphate content according to Bartlett [11].

HPLC separation of minor components of Hb A and Hb A_2

The HPLC system was analogous to that previously described [12–14]. The equipment was comprised of a Beckman gradient HPLC system with Model 110A pumps (Altex), a Model 160 detector and a Model 421 microprocessor controller. A Hewlett-Packard Model 3390A recorder–integrator was also used with this system. A SynChropak CM 300 cation-exchange column of 25 cm × 4.1 mm and 10 μm particle size was obtained from SynChrom (Linden, IN, U.S.A.). The following developers were used: developer A: 30 m*M* Bis–Tris–1.5 m*M* potassium cyanide, pH adjusted to 6.4 with acetic acid; developer B: 30 m*M* Bis–Tris–1.5 m*M* potassium cyanide–0.15 *M* sodium acetate, pH adjusted to 6.4 with acetic acid. The column was preceded by a guard column (5 cm × 4.1 mm) packed with a similar resin (CSC packing material from SynChrom). The column was equilibrated for 20 min with 90% developer A plus 10% developer B. Sample (20 μl) containing 200 μg Hb was injected and Hb A_2 components were eluted by setting a linear gradient of sodium acetate (10% → 80% B for 110 min). Analyses were carried out at a flow-rate of 1 ml/min at room temperature and the pressure of the column was 70 bar; wavelength 405 nm; 0.02 a.u.f.s. and 0.1 cm/min chart speed. Minor components of both Hb A_2 and Hb A were separated using essentially the same gradient system. After the completion of the chromatogram in about 70 min the column was purged with 100% B for 10 min before re-equilibration.

Affinity chromatographic quantitation of total glycosylated hemoglobin

Glycosylated Hb of the whole blood samples was determined by phenylboronate-Sepharose affinity chromatography [15, 16]. Microcolumns prepacked with the resin were obtained from Isolab (Akron, OH, U.S.A.) and the methodology recommended by the company was followed.

HPLC separation of the globin chains of Hb A_2 and its minor components

Globin chain separation by reversed-phase HPLC [17] was used to examine the purity of the isolated Hb A_2 and of Hb A_2 minor components. About 100 g of the original Hb A_2 isolated by DEAE-cellulose chromatography from representative samples and pooled Hb A_{2Ic}, Hb A_{2Id} and Hb A_{2o} fractions were injected onto a C_4 reversed-phase column (Vydac, Hesperia, CA, U.S.A.) developed with a gradient HPLC system. The two developers used had the following compositions: developer B: 0.1% trifluoroacetic acid in water–0.1% trifluoroacetic acid in acetonitrile (40:60); developer A: 0.1% trifluoroacetic acid in water–0.1% trifluoroacetic acid in acetonitrile (80:20). A linear gradient of acetonitrile was supplied with 49% → 61% B set for 70 min. Separation of heme and β, δ, α, $^{G}\gamma$, and $^{A}\gamma$ chains was achieved in this manner. Absorbances at 214 nm were recorded and integrated.

RESULTS

Fig. 1 shows the Bio-Rex 70 chromatographic separation of the minor components of Hb A_2, namely Hb A_{2Ia+b} and Hb A_{2Ic} in a normal adult and a diabetic patient. Both of these components were increased about two-fold in the diabetic patient. About 25 mg of Hb A_2 isolated from 1000 mg

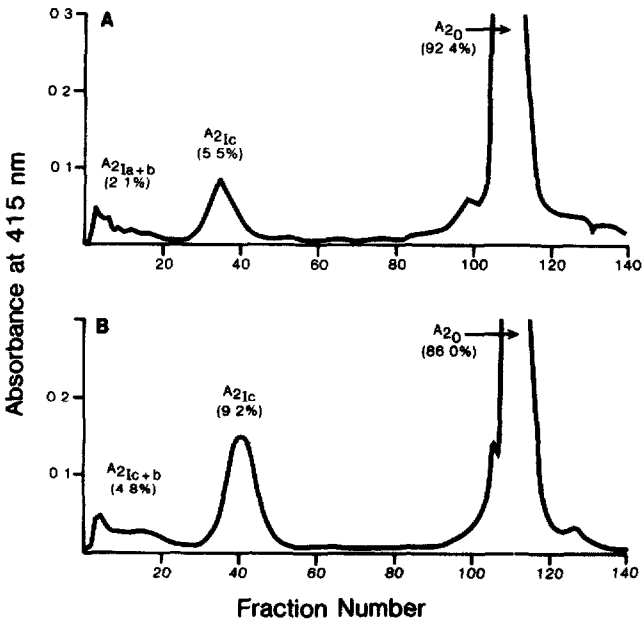

Fig. 1. Bio-Rex 70 chromatographic separation of the glycosylated minor hemoglobins of Hb A_2 in a normal adult (A) and in a diabetic patient (B). About 100 mg of Hb A_2 isolated by DEAE-cellulose chromatography was chromatographed on 25 × 2 cm columns and developed with a linear sodium phosphate gradient.

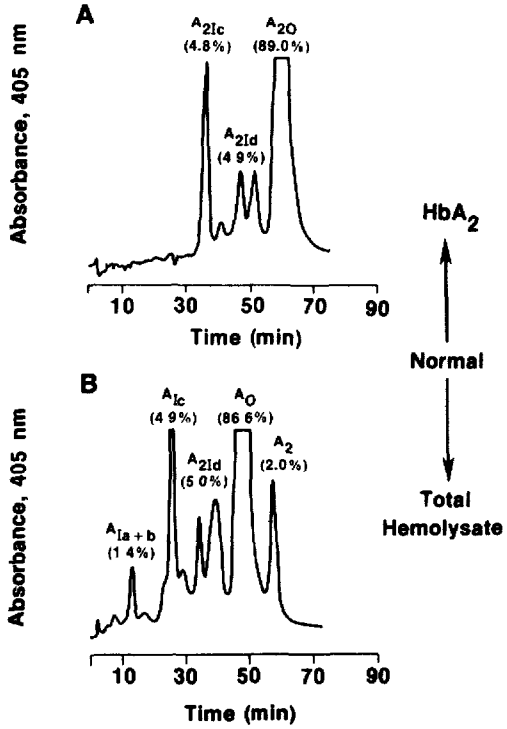

Fig. 2. HPLC separation of the minor components of Hb A_2 from a normal adult (A) and of the hemoglobin minor components in the whole red cell hemolysate from the same person (B). The sample (200 μg) was injected on a cation-exchange HPLC column and developed with a linear sodium acetate gradient (see the text for further details).

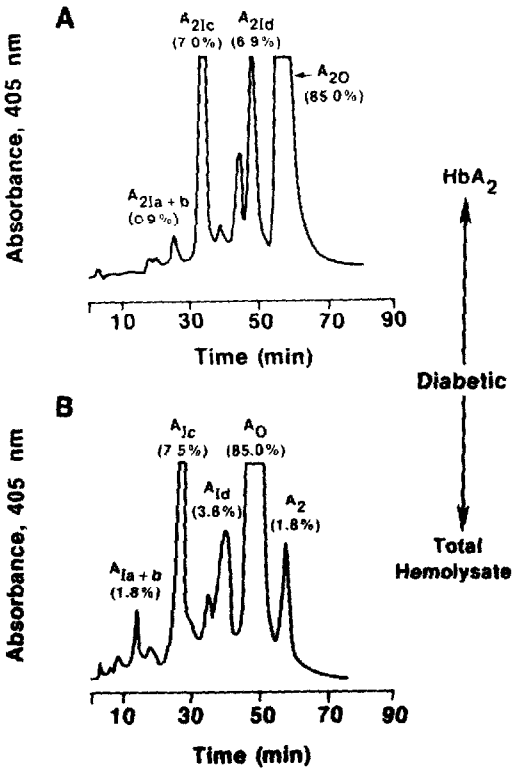

Fig. 3. HPLC separation of the minor components of Hb A_2 from a diabetic patient (A) and of the minor components of Hb A in the whole hemolysate of the same patient (B).

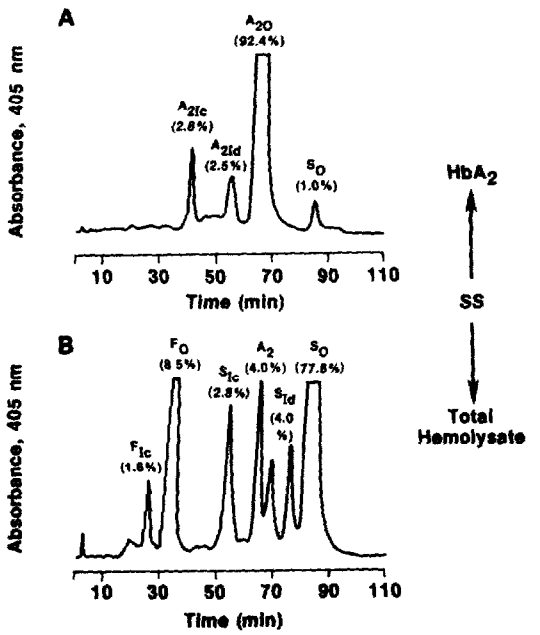

Fig. 4. HPLC separation of the minor components of Hb A_2 (A) and the various Hb components in the whole hemolysate (B) of a sickle cell anemia (SS) patient.

hemolysate were needed to set up each column. Several such columns were run and the hemoglobin components were pooled and concentrated for subsequent chemical analyses. Hb A_{2Ia+b} and Hb A_{2Ic} contained about 15 mmol of 5-hydroxymethylfurfural (5-HMF) per mg of Hb, whereas Hb A_{2o} fraction contained only 1.0 μmol 5-HMF. Analysis for protein-bound phosphate showed about 4 mol of phosphate per mol of Hb A_{2Ia+b}, while Hb A_{2Ic} and Hb A_{2o} contained very little phosphate.

Minor components of Hb A_2, designated Hb A_{2Ia+b}, Hb A_{2Ic} and Hb A_{2Id} were separated by HPLC from normal controls, diabetics and from persons with various hemoglobinopathies. Examples of separation are given in Figs. 2—7. Each figure also has a chromatogram of the whole hemolysate from the same sample. Figs. 2A and 3A depict the HPLC separations of the minor components of Hb A_2 obtained for a normal adult and a diabetic patient, respectively. Both Hb A_{2Ia+b} and Hb A_{2Ic} fractions were increased in the diabetic patient. A group of minor hemoglobin components, namely Hb A_{2Id}, were eluted after Hb A_{2Ic}. Separations of Hb A_{Ia+b}, Hb A_{Ic} and Hb A_{Id} in the whole hemolysate samples were similar to those reported before [12—14]; increased levels of Hb A_{Ia+b} and Hb A_{Ic} were present in the diabetic sample.

Samples containing Hb S were obtained from sickle cell anemia (SS) patients and sickle cell trait (AS) subjects; elution profiles are shown in Figs. 4 and 5. As in normal and diabetic samples, separation of Hb A_{2Ia+b}, Hb A_{2Ic} and Hb A_{2Id} was readily possible in both AS and SS samples (Figs. 4A and 5A). An additional peak after Hb A_{2o} was seen in these samples. This peak was identified as Hb S_o which presumably originated from contamination of Hb

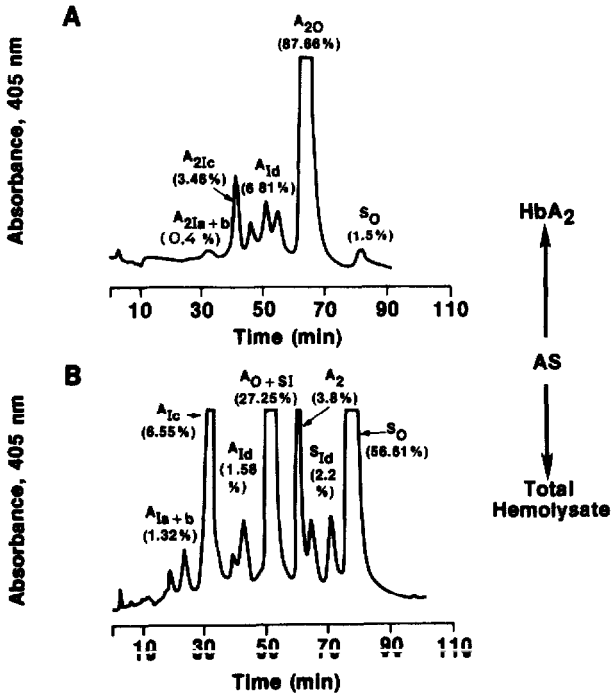

Fig. 5. Separation by HPLC of the minor components of Hb A_2 (A) and of the hemoglobin components in total hemolysate (B) of a sickle cell trait (AS) subject.

A_2 with Hb S during DEAE-cellulose chromatography. As reported before [13], separation of Hb F_{Ic}, Hb F_o and Hb S_{Ic} was possible from the whole red cell lysate of the SS patient (Fig. 4B). In the AS case, Hb A_{Ia+b} and Hb A_{Ic} were readily separated whereas Hb S_{Ic} and Hb A_o co-eluted (Fig. 5B).

In samples containing Hb C, Hb A_2 normally elutes with Hb C on DEAE-cellulose columns. Separation of Hb A_2 and Hb C minor components in a mixture of Hb A_2 and Hb C isolated from an Hb C heterozygote (AC) are illustrated in Fig. 6A; both Hb A_{2Ic} and Hb C_{Ic} could be readily separated. Separation of the minor components Hb A_{Ic} and Hb C_{Ic} and the major components Hb A_2, Hb A_o, and Hb C_o was possible when whole hemolysate was used (Fig. 6B).

Fig. 7 shows the separation of the Hb A_2 minor components and the minor components of Hb A in a β-thalassemia heterozygote. Hb A_{2Ic} level (4.5%) was normal whereas Hb A_{Ic} in the whole blood sample was apparently elevated (7.2%). This is due to the fact that Hb A_{Ic} elutes with Hb F, that is usually increased in β-thalassemia. As expected for a β-thalassemic heterozygote, the level of Hb A_2 was also elevated in this sample (4.9%).

The purity of the Hb A_2 fraction isolated by DEAE-cellulose chromatography and of the minor components of Hb A_2 isolated by HPLC was confirmed by separation of the globin chains with 100 μg of each fraction by reversed-phase HPLC. The results indicated that the Hb A_2 fraction was generally contaminated with about 3.5% Hb A. However, this small amount of Hb A was not contaminating to any extent Hb A_{2Ia+b}, Hb A_{2Ic} or Hb A_{2o}. Hb A_{2Id},

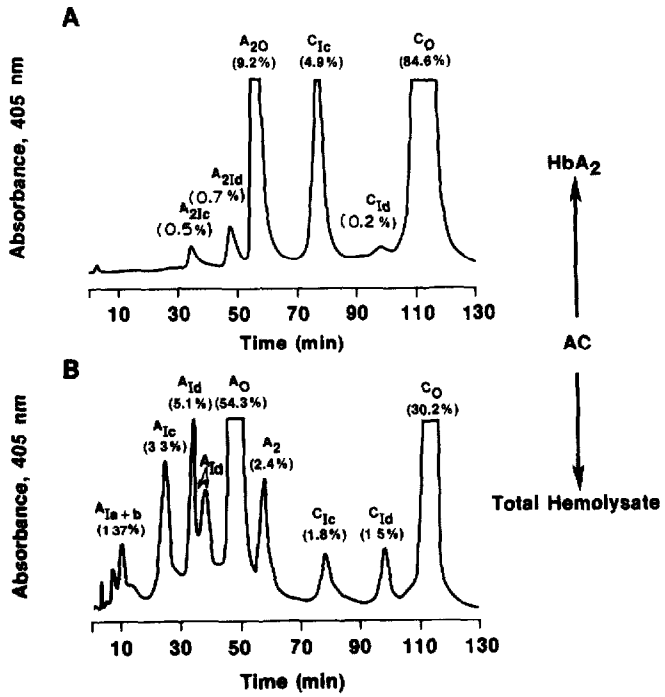

Fig. 6. HPLC separation of the minor components formed from Hb A_2 and Hb C in a mixture of these two hemoglobins (A) and the hemoglobins in whole red cell hemolysate (B) from a Hb C heterozygote (AC).

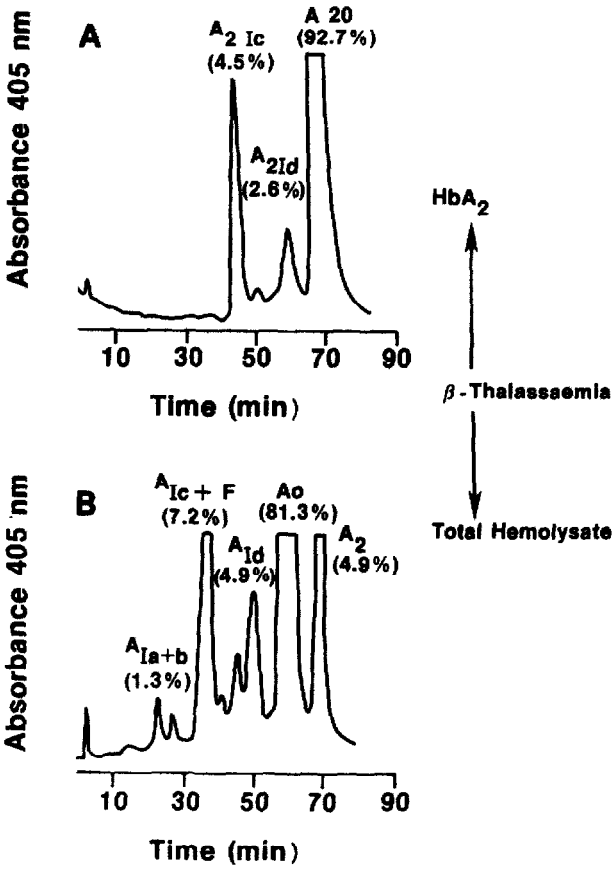

Fig. 7. HPLC separation of Hb A_2 minor components (A) and the minor hemoglobins in total hemolysate (B) of a β-thalassemia heterozygote.

TABLE I

SUMMARY OF THE RESULTS

The Hb A_{Ic}, Hb A_{2Ic}, Hb S_{Ic} and Hb C_{Ic} levels were calculated as percentages of the total Hb A, Hb A_2, Hb S and Hb C, respectively. Values are given as mean ± S.D.

Condition	*n*	Hb A_{Ic} (%)	Hb A_{2Ic} (%)	Total glycosylated Hb (%)	Hb S_{Ic} (%)	Hb C_{Ic} (%)
Normal	10	4.54 ± 0.25	4.24 ± 0.51	4.39 ± 0.42	—	-
Diabetic	23	9.36 ± 2.15	8.72 ± 2.12	11.91 ± 3.24	—	—
Sickle cell anemia	14	—	2.98 ± 1.29	3.21 ± 0.62	3.29 ± 0.91	—
Sickle cell trait	5	4.75 ± 0.49	4.21 ± 0.55	4.65 ± 0.2	—	—
SC disease	5	—	2.84 ± 0.26	4.0 ± 0.31	—	3.13 ± 0.30
Hb C trait	2	4.89 ± 0.1	4.35 ± 0.57	5.80 ± 0.40	—	5.0 ± 0.50
β-Thalassemia heterozygote	4	—	4.15 ± 0.70	5.28 ± 0.42	—	—

on the other hand, appeared to have Hb A contamination. This was not surprising because under the described chromatographic conditions Hb A_0 and Hb A_{2Id} had similar retention times.

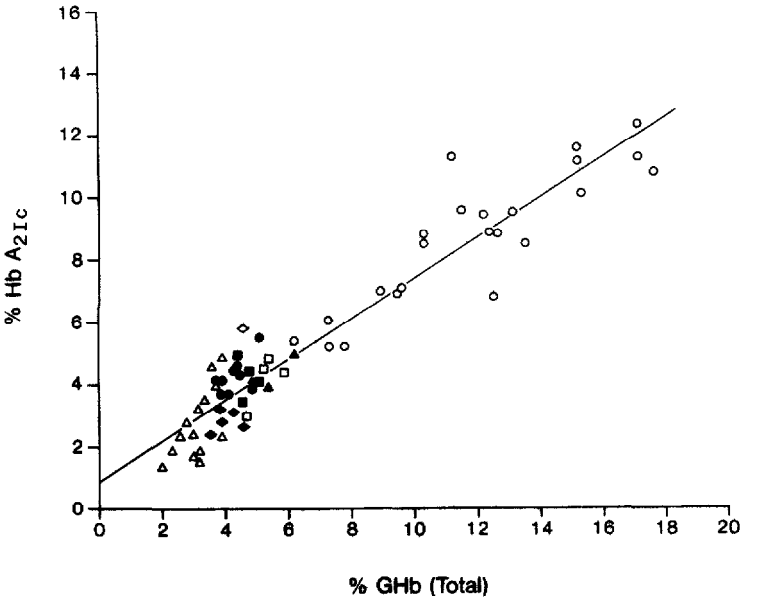

Fig. 8. Correlation of Hb A_{2Ic} levels with the levels of total glycosylated hemoglobin (GHb) determined by affinity chromatography using red blood cell samples from normal controls, diabetics and in persons with common hemoglobinopathies. •, Normal; ○, diabetic; △, SS; ◇, S-$\delta\beta$-thalassemia; ▪, AS; ♦, SC; ▲, AC; □, A-β-thalassemia. $y = 0.65x + 0.98$; $n = 61$; $r = 0.95$.

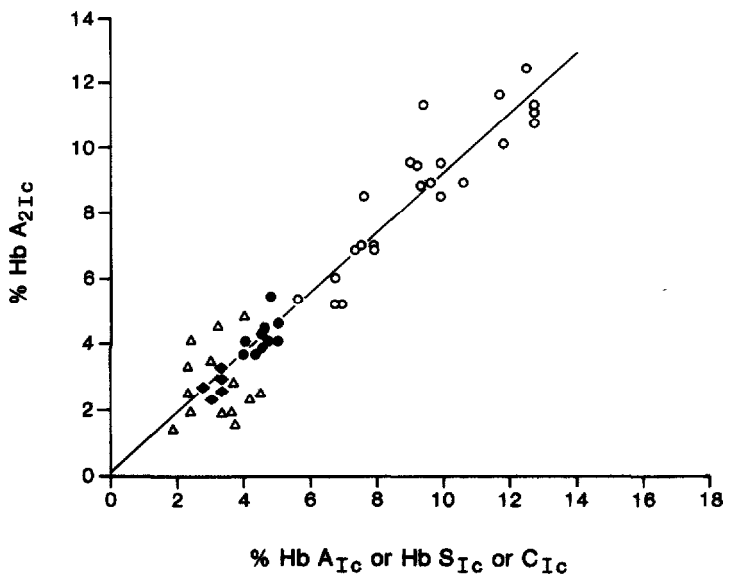

Fig. 9. Correlation of Hb A_{2Ic} levels with the levels of Hb A_{Ic} in normals and diabetics, Hb S_{Ic} in sickle cell anemia patients and of Hb C_{Ic} in SC patients. The percentages of Hb A_{Ic}, Hb S_{Ic} and Hb C_{Ic} were calculated as percentages of total Hb A, Hb S and Hb C, respectively. •, Normal; ○, diabetic (A_{Ic}); △, SS (S_{Ic}); ♦, SC (C_{Ic}). $y = 0.91x + 0.13$; $n = 37$; $r = 0.95$.

Hb A_{2Ic} values were compared with other conventional methods of determining the glycosylated hemoglobins such as Hb A_{Ic} and the total glycosylated Hb. Fig. 8 shows the linear regression for Hb A_{2Ic} and glycosylated Hb and Fig. 9 for Hb A_{2Ic} and Hb A_{Ic}, Hb S_{Ic} or Hb C_{Ic} (Hb S_{Ic} and Hb C_{Ic} were expressed as percentage of total Hb S or Hb C). The correlation in both instances were excellent with a γ value of 0.95. Table I summarizes the levels of Hb A_{Ic} (Hb S_{Ic} or Hb C_{Ic}), Hb A_{2Ic} and glycosylated Hb in normals, diabetics and in various hemoglobinophathies.

DISCUSSION

Determination of Hb A_{Ic} or Hb A_I has been generally used as a tool for monitoring glycemic control in diabetic patients [3]. Affinity chromatography has been recently introduced to quantitate total glycosylated hemoglobin [15, 16]. However, many laboratories have not yet switched to this method probably because they believe that looking at one specific type of glycosylated hemoglobin, say Hb A_{Ic}, would be more meaningful rather than a sum of a variety of glycosylated hemoglobins. Accurate quantitation of Hb A_{Ic} poses a problem in the presence of fetal hemoglobin, Hb H in α-thalassemia and in the presence of abnormal hemoglobins including the common variants S and C and some rare variants N-Baltimore and J-Baltimore [3]. In this study we have shown that quantitation of Hb A_{2Ic} is an alternative to Hb A_{Ic} determination, which can be achieved with precision by HPLC. The above mentioned interferences in the Hb A_{Ic} determination should not influence the Hb A_{2Ic} quantitation because Hb A_2 can be prepared in relatively pure form even in the presence of these apparent interferences. The correlation between glycosylated Hb and Hb A_{2Ic} and Hb A_{Ic} (Hb S_{Ic} or Hb C_{Ic}) and Hb A_{2Ic} are excellent and thus Hb A_{2Ic} levels should be as acceptable as the other two parameters. The labile glycosylated hemoglobin (the Schiff base) also appears to be eliminated during the Hb A_2 isolation by DEAE-cellulose chromatography. Our results suggest that the determination of glycosylated Hb A_2 has some apparent advantages over the current determination of Hb A_{Ic} or Hb A_I. This method, however, is labor-intensive, needs special equipment and requires good technical skill.

HPLC separation of Hb A_2 minor components showed lower than expected (from Bio-Rex 70 separations) levels of Hb A_{2Ia+b}. In fact, in many instances Hb A_{2Ia+b} was not present in detectable quantities in normals. We have observed a similar disparity between Bio-Rex 70 and HPLC separation of Hb A_{Ia+b} before [14]. It is also possible that some of the Hb A_{2Ia+b} was retained on the DEAE column during Hb A_2 isolation step. The HPLC chromatogram of whole red cell hemolysate from normal adults and diabetics showed separation of Hb $A_{Id1(s)}$. Hb A_{Id1}, Hb A_{Id2}, and Hb A_{Id3} were separated before by Bio-Rex 70 chromatography and Hb A_{Id3} was identified as the minor Hb formed by glycosylation of the α-chain NH_2-terminus and Hb A_{Id1} as that formed by reaction with oxidized glutathione [5]. The minor components Hb $A_{2Id1(s)}$ are most likely synonymous to Hb $A_{Id1(s)}$ and may be termed Hb A_{2Id1}, Hb A_{2Id2} and Hb A_{2Id3} in the order in which they are separating. Interestingly, when the proportions of Hb A_{Id3} and Hb A_{2Id3} were calculated, they were

significantly increased in the diabetic patients (Hb A_{Id3} = 2.53 versus 3.59% and Hb A_{2Id3} = 2.45 versus 3.70%). Similar increases in Hb A_{Id3} in diabetic patients were also reported before [5].

The level of Hb A_{2Ic} was nearly the same in Hb C, Hb S and β-thalassemia heterozygotes as in normal controls, but lower in SS and SC patients. Hb S_{Ic} (of total Hb S) and the total glycosylated Hb were also lower in these patients. This is due to the fact that these patients have hemolytic anemia. Moreover, good correlation of Hb A_{2Ic} with Hb A_{Ic}, Hb S_{Ic} and Hb C_{Ic} strongly indicate that these normal and abnormal hemoglobins are indeed being glycosylated at the same rate in vivo and the concentrations of these glycosylated components are influenced primarily by the factors such as diabetes and hemolytic anemia.

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