Evaluation of HbA_{1c} Determination Methods in Patients With Hemoglobinopathies

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OBJECTIVE — To evaluate commercially available determination methods for HbA_{1c} in patients with hemoglobin variants.

RESEARCH DESIGN AND METHODS — HbA_{1c} values were determined with various commercially available methods, including ion-exchange high-performance liquid chromatography (HPLC), boronate affinity assay, and immunoagglutination in patients with the hemoglobin mutations Hb Graz, Hb Sherwood Forest, Hb O Padova, Hb D, and Hb S.

RESULTS — The effect of hemoglobinopathies on glycohemoglobin measurements was highly method dependent. The HPLC methods for HbA_{1c} determination lacked the resolution necessary to differentiate hemoglobin variants. They demonstrated additional peaks in the chromatograms and HbA_{1c} results either too low or too high compared with the nondiabetic reference range. With all immunoassays, Hb Graz demonstrated falsely low values. The other hemoglobinopathies in our study caused falsely low and/or high HbA_{1c} results in immunoagglutination methods. The boronate affinity method showed values in an acceptable range for all hemoglobin variants.

CONCLUSIONS — Because of the local occurrence of Hb variants and the ethnic origin of a given population, every individual laboratory must establish and validate its own assay method. In managing diabetic patients, knowledge of hemoglobinopathies influencing HbA_{1c} determination methods is essential because hemoglobin variants could cause mismanagement of diabetes resulting from false HbA_{1c} determinations.

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Measurement of HbA_{1c} in diabetic patients is an established procedure for evaluating long-term control of diabetes. HbA_{1c} is a stable minor glycated subfraction of hemoglobin and was originally identified with cation-exchange chromatography (1). The Diabetes Control and Complications Trial (DCCT) confirmed the direct relationship between the degree of glycemic control as estimated by glycohemoglobin determinations and the development and progression of long-term complications in type 1 diabetes (2). Because different commercially available assays measure different fractions of glycated hemoglobin, results may vary depending on the method used. Despite advances in the standardization of methods for glycohemoglobins (3), an increasing number of hemoglobinopathies cause false results in HbA_{1c} determinations.

The aim of our study was to evaluate commercially available determination methods for glycated hemoglobin in patients with hemoglobin variants. These included automated high-performance liquid chromatography (HPLC), boronate affinity binding assay, and immunoagglutination in patients with the hemoglobin variants Hb Graz, Hb Sherwood Forest, Hb O Padova, Hb D, and Hb S.

RESEARCH DESIGN AND METHODS

We investigated HbA_{1c} determination methods in one type 2 diabetic and two nondiabetic patients with the hemoglobin variant Hb Graz [$\alpha_2\beta_2$2(NA2) His Leu] (4), one nondiabetic patient with the mutant hemoglobin Hb Sherwood Forest [$\alpha_2\beta_2$104(G6)Arg Thr] (5), and one type 2 diabetic patient with Hb D [$\alpha_2\beta_2$121(GH4) Glu Gln] (6). Two patients, one of whom has type 1 diabetes, carry the α-chain variant Hb O Padova [$\beta_2\alpha_2$30(B11)Glu Lys] (7). All of these hemoglobin variants are clinically silent hemoglobin mutations detected in Caucasian individuals. Amino acid analysis and sequence analysis of DNA were performed as described previously (4–7), and the routine hematologic data were in the normal range. Three patients were of African origin and had sickle cell anemia [Hb S (β6Glu Val)]. In one homozygous patient (Hb S patient 1), hemoglobin electrophoresis demonstrated the following: Hb S 91.9%, HbA_2 5.2% (normal 1.5–4), HbA 0.0% (normal 96–98), anemia with 3.3×10^{12}/l erythrocytes (normal 3.8–5.5), hemoglobin 4.9 mmol/l (normal 7.4–11.2), and hematocrit 24.5% (normal 36–53). Hb S, HbA_2, and HbA were 29.3, 2.4, and 68.3%, respectively, in one diabetic patient (Hb S patient 2), and 32.3, 2.5, and 65.3% in the other nondiabetic patient (Hb S patient 3) with heterozygous sickle cell disease (8). Routine hematologic data were normal in both Hb S patient 2 and Hb S patient 3.

The determinations of HbA_{1c} with all methods were performed on EDTA-anticoagulated blood samples within 3 days. All HbA_{1c} determinations were analyzed blindly in triplicate by experienced laboratory personnel, and procedures were in accordance with the principles of the Declaration of Helsinki and the local ethics committee recommendations. The Austrian laboratories

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Abbreviations: DCCT, Diabetes Control and Complications Trial; HPLC, high-performance liquid chromatography.
A table elsewhere in this issue shows conventional and Système International (SI) units and conversion factors for many substances.

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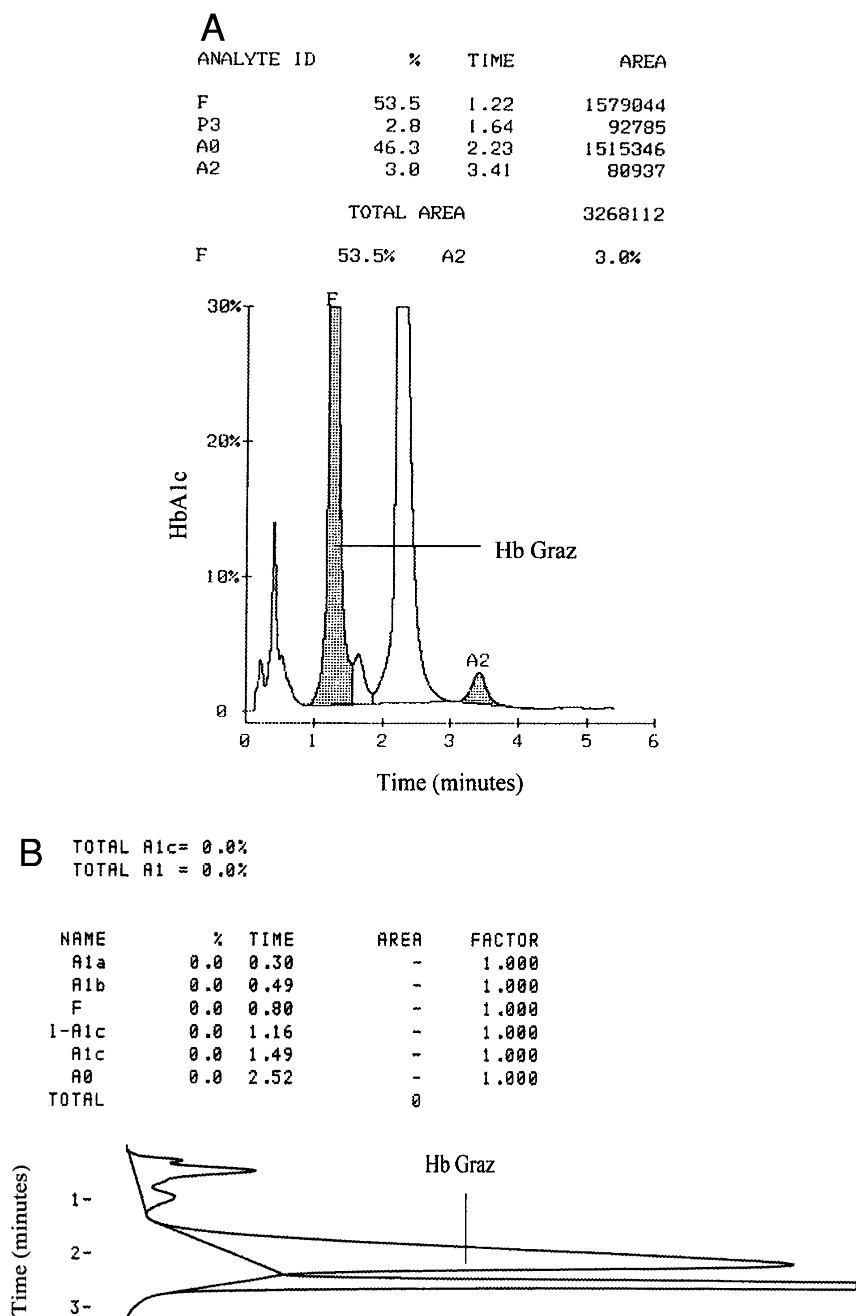

Figure 1—A: Hemoglobin separation obtained with the Bio-Rad Variant using the β-Thalassemia Short Program in a patient with Hb Graz. B: Chromatogram from the ion-exchange analyzer Merck Hitachi L-9100 for a patient with Hb Graz.

involved participate in the Austrian Program for Quality Assurance and Standardization of Medical-Diagnostic Examinations. Calibration of all instruments was done according to the manufacturers' instructions.

Fasting blood glucose was determined with a hexokinase/glucose-6-phosphate dehydrogenase colorimetric method (Gluco-Quant; Roche, Vienna, Austria), and the fructosamine assay was determined with a colorimetric test using nitroblue tetrazolium in alkaline solution (Unimate FRA; Roche).

The Diamat HPLC (Bio-Rad, Vienna, Austria) is the routine method used by the DCCT and involves a buffer containing borate and a step gradient of three phosphate buffers with increasing ionic strength. The automated ion-exchange HPLC Variant (Bio-Rad, Munich, Germany) involving the β-Thalassemia Short Program separates hemoglobin components chromatographically with two phosphate buffers that differ in pH and ionic strength. Hemoglobins then pass the flow cell of a filter photometer, and changes in absorbance are measured. The automated HPLC ion-exchange analyzer Merck Hitachi L-9100 (Merck, Vienna, Austria) uses gradients of phosphate buffers to separate the hemoglobin fractions. The automated HPLC method Hi-Auto $A1_{1c}$, HA-8140 (Menarini, Florence, Italy) uses cation-exchange and reversed-phase chromatography on a solid phase of methacrylic acid and methacrylate ester. The hemoglobin fractions are eluted by a varying pH in the mobile phase.

The automated Abbott IMx glycated hemoglobin test (Abbott, Vienna, Austria) is an ion-capture boronate affinity binding assay based on the specific complex formation of the glucose cis-diol group and 3-aminophenylboronic acid. The DCA 2000 (Bayer, Vienna, Austria) is an immunologic method that uses a specific antibody against the first six amino acid residues of the glycated NH_2-terminal of hemoglobin. HbA_{1c} causes inhibition of agglutination, and decreasing absorbances are measured. The Cobas Integra (Roche) uses monoclonal antibodies attached to latex particles. The measured change in turbidity is inversely correlated with the amount of bound glycopeptides. The Tina-Quant hemoglobin A_{1c} test, which was performed on a Hitachi 911 (Roche), is an immunoturbidimetric test in which anti-HbA_{1c} antibodies produce a soluble immunocomplex with HbA_{1c}. The resulting agglutinated complex is measured turbidimetrically. The A1c At Home Test Kit (Becton Dickinson, Rungis, France) determines HbA_{1c} from a dried blood sample using the Unimate diagnostic reagent system with a Cobas Mira (Roche) to perform a turbidimetric immunoassay with monoclonal antibodies to glycated hemoglobin.

RESULTS

Hb Graz

In diabetic and nondiabetic patients, this mutant hemoglobin caused extremely high HbA_{1c} results with the Bio-Rad Diamat (9). In the β-Thalassemia Short Program with the Bio-Rad Variant, Hb Graz migrated with fetal Hb (Fig. 1A), again with very high results (Table 1). The Merck HPLC chromatogram showed a double peak at HbA_0, but calculation with this method

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Table 1—HbA_{1c} results in patients with hemoglobin variants

Hemoglobin variant	FBG (mmol/l)	Fructosamine (mmol/l)	HbA_{1c}(%)								
			IMx	HPLC 1	HPLC 2	HPLC 3	HPLC 4	DCA	Integra	Tina	Unimate
Nondiabetic reference range	<6.1	205–285	4.4–6.4	4.1–6.2	3.8–6.1	3.6–5.7	4.5–5.7	4.5–5.7	4.5–5.7	4.8–6.0	4.5–5.7
Hb Graz 1	9.0	303	6.7	48.3	53.7	No result	Abn. sep.	4.9	5.2	5.6	5.7
Hb Graz 2	4.4	233	5	48.6	52.5	No result	Abn. sep.	3.9	4.2	4.7	4.4
Hb Graz 3	4.4	238	4.6	48.3	52.9	No result	Abn. sep.	3.4	3.9	4.5	4.1
Hb Sherwood Forest	5.0	224	4.5	2.2/49.5	49.2	1.2	Abn. sep.	4.9	5	5.5	5.4
Hb O Padova 1	3.4	455	8.4	8.9	7.1	5.8	10.3 (var. Hb)	9.2	9.4	9.3	10.1
Hb O Padova 2	3.8	253	5.7	5.3	4.4	3.7	6.6 (var. Hb)	5.3	5.5	5.7	5.9
Hb D	6.0	273	5.3	3.4	3.9	2.9	7.6 (var. Hb)	5.7	4.6	4.6	6.6
Hb S 1	5.8	251	5.1	0	0.3	0	Abn. sep.	3.5	6	6.1	5.2
Hb S 2	4.8	282	5.7	6.1	4.1	3.1	7.1 (var. Hb)	5.5	6.7	6.1	7.4
Hb S 3	6.2	233	6.1	6.1	3.6	2.9	7.1 (var. Hb)	5.7	6.9	6	7.8

Abn. sep., abnormal separation; var. Hb, variant hemoglobin; IMx, IMx glycated hemoglobin test (Abbott); HPLC 1, Diamat (Bio-Rad); HPLC 2, Variant (Bio-Rad); HPLC 3, Hitachi L-9100 (Merck); HPLC 4, Hi-Auto A_{1c}, HA-8140 (Menarini); DCA, DCA 2000 (Bayer); Integra, Cobas Integra (Roche); Tina, Tina-Quant Hitachi 911 (Roche); Unimate, A1c At Home Test Kit and Cobas Mira (Becton Dickinson and Roche, respectively).

failed, and the percentage for any peak of the chromatogram was 0 (Fig. 1B). The chromatogram of Hb Graz performed with HPLC from Menarini has been published previously, and this method gave the result as "abnormal separation" (9). The results of the Abbott IMx appeared to be in a reasonable range compared with the fructosamine and blood glucose values. All of the immunological methods (DCA 2000, Cobas Integra, Tina-Quant Hitachi 911, and the Unimate Cobas Mira) showed values below the nondiabetic reference value for both of the nondiabetic patients with Hb Graz (Table 1).

Hb Sherwood Forest
The HPLC chromatogram with the Bio-Rad Diamat for Hb Sherwood Forest has been published elsewhere (5). Using the Diamat, we observed that this hemoglobin variant caused results that were either extremely higher than or below the nondiabetic reference, depending on the inclusion of the additional peak in the HbA_{1c} peak calculation (Table 1). In the β-Thalassemia Short Program with the ion-exchange HPLC Variant, Hb Sherwood Forest migrated like HbA_{1c}, and the results were extremely high (Fig. 2A). The Merck HPLC chromatogram showed a double peak at HbA_0, but calculation with this method failed, and falsely low values were given (Fig. 2B). The chromatogram result of Hb Sherwood Forest performed with the HPLC from Menarini was "abnormal separation" (10). The HbA_{1c} results of the Abbott IMx and of all the immunoagglutination methods showed values within the nondiabetic reference range (Table 1).

Hb O Padova
We examined one type 1 diabetic and one nondiabetic patient with this mutation. The HPLC chromatogram with the Bio-Rad Diamat has been published previously (7) and showed a double peak at HbA_0. The chromatogram of the β-Thalassemia Short Program with the Bio-Rad Variant had several "unknown" peaks, including an S window. The HbA_{1c} values seemed too low compared with the fructosamine or the HbA_{1c} results obtained with other methods (Fig. 3A). The Merck HPLC chromatogram showed a double peak at HbA_0, and the peak calculations of this method showed low HbA_{1c} results within the nondiabetic reference range for both of the patients with Hb O Padova (Table 1). The chromatogram of Hb O Padova performed with the HPLC from Menarini gave a result of "variant Hb" and demonstrated a double peak at HbA_0 (Fig. 3B). The HbA_{1c} results obtained with the Abbott IMx for the diabetic and the nondiabetic patients demonstrated a difference of 2.7% compared with the immunoagglutination methods that showed a mean difference of 3.6% (Table 1).

Hb D
The examined blood of the patient with Hb D showed a normal chromatogram with the Bio-Rad Diamat, but the HbA_{1c} values were below the nondiabetic reference range (Table 1). The β-Thalassemia Short Program with the Bio-Rad Variant demonstrated Hb D with the previously published D window (11). The Merck HPLC chromatogram did not recognize the hemoglobin variant with an additional peak but showed HbA_{1c} results below the nondiabetic range (Table 1). The chromatogram of Hb D performed with the HPLC from Menarini gave the result "variant Hb" and demonstrated a double peak at HbA_0 (10). The HbA_{1c} values performed with the Cobas Integra and Tina-Quant Hitachi 911 were in the low nondiabetic range, with the Unimate Cobas Mira above the nondiabetic reference range. The HbA_{1c} results of the Abbott IMx and the DCA 2000 were within the nondiabetic range (Table 1).

Hb S
The samples of the three patients with Hb S examined with the Bio-Rad Diamat showed an additional peak at HbA_0 called "S/A0." The HbA_{1c} values for the anemic Hb S patient 1 were clearly below the nondiabetic reference range with the Bio-Rad Diamat and were in the upper range of normal for both of the other patients with Hb S. The chromatograms with the β-Thalassemia Short Program with the Bio-Rad Variant demonstrated the S window (11), and the HbA_{1c} results for all three of the patients were below the nondiabetic reference range (Table 1). The Merck HPLC chromatogram showed results below the nondiabetic range, and Hb S was not recognized in the chromatogram. The chromatograms of Hb S performed with the HPLC from Menarini resulted in "abnormal separation" in the anemic patient (Hb S

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A

ANALYTE ID	%	TIME	AREA
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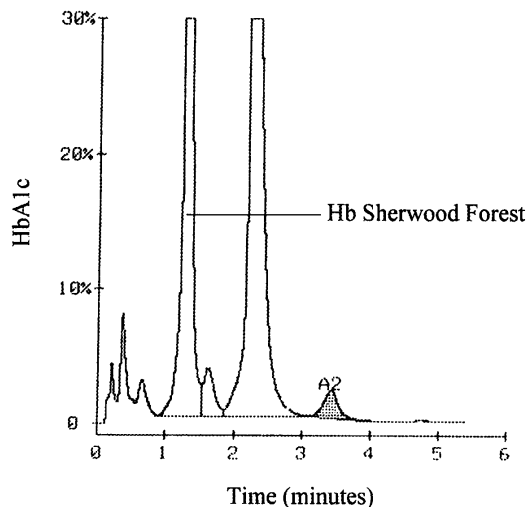

B

TOTAL A1c= 1.2%
TOTAL A1 = 4.8%

NAME	%	TIME	AREA	FACTOR
A1a	0.8	0.25	13481	1.000
A1b	2.8	0.40	48226	1.000
F	0.4	0.76	7793	1.000
1-A1c	0.0	1.18	-	1.000
A1c	1.2	1.48	20900	1.000
A0	94.8	2.52	1652273	1.000
TOTAL			1742673	

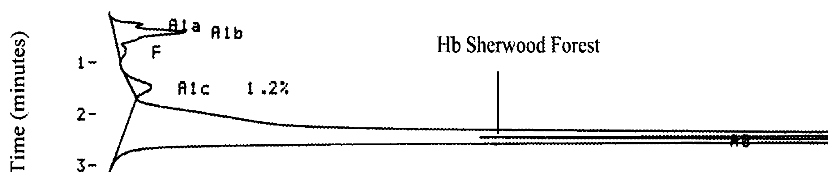

Figure 2—A: Hemoglobin separation obtained with the Bio-Rad Variant using the β-Thalassemia Short Program in the patient with Hb Sherwood Forest. B: Chromatogram from the ion-exchange analyzer Merck Hitachi L-9100 for the patient with Hb Sherwood Forest.

patient 1) and "variant Hb" in both of the other patients (10). In all patients with Hb S, the immunoturbidimetric tests showed values above the nondiabetic reference range, and the HbA_{1c} results performed with the Abbot IMx were within the nondiabetic range. The HbA_{1c} results of DCA 2000 were below the reference range in the anemic patient and were within the nondiabetic range for both of the other patients (Table 1).

CONCLUSIONS

The first clinically useful method for determining HbA_{1c} levels was published in 1978 (12). Since the publication of the DCCT, which used the Bio-Rad Diamat HPLC as the HbA_{1c} determination method, HbA_{1c} results have become increasingly important in the treatment of diabetes (13). An increasing number of requests highlights the need for high-quality and reliable assays. So far, no universally accepted methodologic gold standard exists. Despite advances in the standardization of methods for glycohemoglobins, an increasing number of hemoglobinopathies cause false results in HbA_{1c} determinations because interference from hemoglobin variants is a limitation of each method and cannot be altered by standardization efforts.

In patients without hemoglobin variants, the HbA_{1c} values are highly sensitive in reflecting elevations of blood glucose. A deviation of 1% in HbA_{1c} results in a change of 1.4–1.9 mmol/l in an average blood glucose concentration. We have known for some time that the NH_2-terminal valine residue of the β-globin chain can be glycated. "Hemoglobin A_{1c}" was originally a term for an ion-exchange chromatographic peak and is now defined as irreversibly glycated hemoglobin molecules at one or both NH_2-terminal valines of the β-chains. The actual extent of glycation and the relative involvement of the α- and β-chains, however, still remain unclear (1). The ion-exchange HPLC methods for HbA_{1c} determination usually indicate the presence of a variant, but they lack the resolution necessary to differentiate hemoglobin variants. They demonstrate additional peaks in the chromatograms combined with clinically low or high results (Table 1). One HPLC method (Hi-Auto A_{1c}, HA-8140) has separation conditions that seem to detect all mutant hemoglobins (10). The HbA_{1c} results with this method and the clinical picture of diabetes in our study, however, do not match. As demonstrated in Hb S patient 1, sickle cell anemia causes decreased erythrocyte survival and thereby decreased exposure time of hemoglobin to glucose, which results in a decreased percentage of HbA_{1c} undergoing glycation (Table 1). Hemoglobin mutations at the NH_2-terminal of the β-chain cause HbA_{1c} values below the nondiabetic reference range in immunoagglutination (14). In our study of patients with Hb Graz, all the immunoassays produced falsely low values, which confirms that the ability of the monoclonal antibody to detect the epitope at the NH_2-terminal β-chain is hampered by this hemoglobin variant (14). The use of affinity methods has already been suggested (3) because they mainly measure glycohemoglobin regardless of the glycation site and may be more useful clinically in reflecting glycemic control. The boronate affinity method we used in our study showed values in a clinically reasonable range for all hemoglobin variants.

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A method may give a value in the normal range for a nondiabetic patient with a hemoglobin variant, but this is not an assurance that no interference is present. The interference may be subtle in the normal range but increases with increasing HbA_{1c} (Table 1). This study shows that the effect of certain hemoglobinopathies on HbA_{1c} measurements is highly method dependent. When HbA_{1c} results do not fit the clinical picture, or additional peaks in HPLC chromatograms are displayed, this should be noted, and further investigations are indicated. Recent studies have reported that the use of immunoassays in Hb variants may give false HbA_{1c} results (14–17). These methods, however, do not allow recognition of the variant. If the clinical impression and HbA_{1c} test results do not match, then HbA_{1c} values should be determined with a second method based on a different principle, or fructosamine should be determined as an alternative (9,15). Measurement of fructosamine is used to document the glycemic status over a short period of time (1–2 weeks). Fructosamine results depend on glycation of serum proteins (13) and are not influenced by hemoglobin variants. Because blood glucose measurements and fructosamine results are not always well correlated (18), correct HbA_{1c} and mean blood glucose values are the most appropriate methods but only when clinicians properly account for the contribution of hemoglobin variants. We suggest calculation of mean blood glucose (e.g., a mean of 4–5 daily home measurements for 1 week before HbA_{1c} evaluation) and/or measurement of fructosamine as alternative methods for long-term control of diabetes in patients with hemoglobinopathies (19). The results of our study emphasize the need for additional investigations of the different effects of hemoglobin variants in the numerous commercially available HbA_{1c} assays.

Today, >700 hemoglobin variants are known (20), and some, like Hb S, are reported to be common, with a prevalence of up to 9% in African-Americans (21). We reported an estimated prevalence of silent hemoglobin variants of 0.6‰ (19), but exact prevalence data are still not available. Because of local appearances of Hb variants and the ethnic origin of a population, every laboratory must establish and secure its own assay method for HbA_{1c}. In managing diabetic patients, knowledge of hemoglobinopathies influencing HbA_{1c} determination methods is essential because hemoglobin variants may cause misman-

A

ANALYTE ID	%	TIME	AREA
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A2	1.3	3.75	30317
S-WINDOW	0.7	4.51	18171
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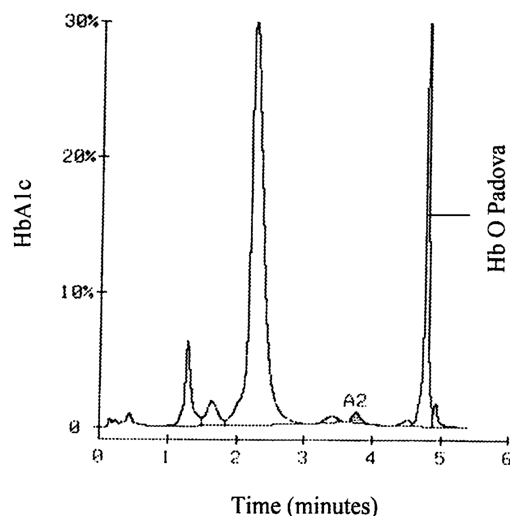

B

HbA1c 10.3 % HbA1 12.7 %
HbF 0.0 %

VARIANT Hb

	TIME	AREA	%
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P2	1'04"	199	0.8
P3	1'14"	203	0.8
P4 A1c	2'06"	2634	10.3
P5 A0	3'14"	22232	87.3

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P6 3'24" 12727

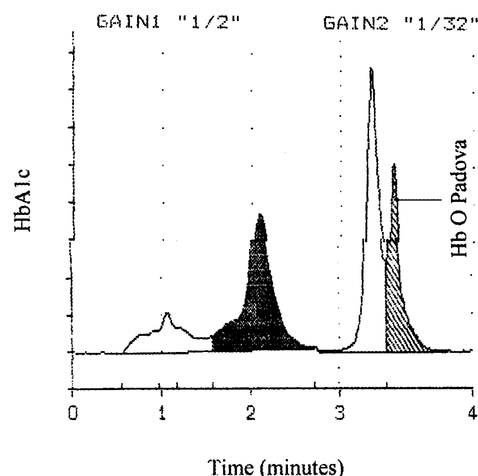

Figure 3—A: Hemoglobin separation obtained with the Bio-Rad Variant using the β-Thalassemia Short Program in the diabetic patient with Hb O Padova. B: Chromatogram with the Hi-Auto A_{1c}, HA-8140 for the diabetic patient with Hb O Padova.

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agement of diabetes resulting from false HbA_{1c} results.

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