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Immunoturbidimetric Assay of Glycated Hemoglobin

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We investigated the performances of HbA1c determination by a latex enhanced turbidimetric immunoassay using the specific monoclonal antibodies (Unimate, Roche) against the β-N-terminal fragments. The coefficients of variation ranges from 1.7 to 3.8% within assay (n = 30) and from 3.9 to 4.9% between assay (n = 20).

The assay was linear from 2.5 to 14.9% of HbA1c. No interferences was found from fetal, carbamylated, or variant (S) hemoglobins and from labile Schiff adduct with glucose. The following relationship was derived from fresh sample comparison between HPLC (Diamat-BioRad) (*x*) and immunoassay (*y*) method: $y = 0.971\ x + 0.87\%$, $r = 0.98$, $n = 115$. The immunoassay provides a highly precise and specific method for HbA1c. J. Clin. Lab. Anal. 13:5–8, 1999.

Key Words: HbA1c; glycemic balance control; glycated hemoglobin-labile fraction; fetal hemoglobin; latex enhanced immunoassay

INTRODUCTION

Glycohemoglobin is widely accepted as an excellent indicator to monitor long-term glycemic balance in diabetics and allows the identification of patients with poor glycemic control and the correct adjustments in therapy (1–3).

The term *glycohemoglobin* refers to the sum of reaction products between the free aldehyde groups of carbohydrates and primary amino groups of hemoglobin chains. The first step of this relatively fast and reversible reaction produces a labile aldimine form which, in a second step, is partially and slowly converted in a stable ketoamine form (4).

Today the analytic methods available allow measurement of total glycohemoglobin or different components such as HbA1 and HbA1c (glucose adduct to N-terminal valine of the β-chain of hemoglobin). Cation-exchange chromatography methods with minicolumns (5) and electroendoosmosis (6) methods were the first to be introduced, but they are subjected to a number of interferences. Further methodological improvements in ion-exchange or affinity HPLC procedures (7,8) led to the development of totally automated HPLC systems dedicated to the HbA1c assay, the glycohemoglobin fraction clinically recommended. Recently, immunoassays using monoclonal antibodies directed against the glycated N-terminal group of hemoglobin β-chain were introduced (9,10). Some of these methods can be performed on common laboratory analyzers without the need of dedicated instrumentation.

The aim of this work was to evaluate analytical performances of an immunoturbidimetric method for HbA1c determination automated on a Cobas Mira Plus analyzer (Roche). Ion-exchange HPLC, probably the widest diffused technique, was chosen as the comparison method due to the lack of a reference method (4).

MATERIALS AND METHODS

This study analyzed 115 EDTA K_3-treated blood samples collected from diabetics and nondiabetics, five subjects with hemoglobin variants, and 20 nondiabetic patients with renal failure before dialysis. Samples were assayed the same day of collection or stored at room temperature and evaluated within three days.

Unimate Method

The Unimate method (Roche, Basel, Switzerland) is a competitive latex agglutination inhibition immunoassay based on monoclonal antibodies against the N-terminal group of β-chain of HbA1c. The blood sample is hemolyzed by a specific reagent and hemoglobin is proteolytically degraded. The β-N-terminal fragments of HbA1c bind the specific monoclonal antibodies while the remaining free antibodies are agglutinated with a synthetic polymer carrying multiple copies of the HbA1c β-N-terminal amino acid structure. The agglutination rate is photometrically determined at 550 nm and inversely related to the HbA1c concentration. At the same

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TABLE 1. Imprecision of Unimate Method

	Within-assay n = 30			Between-assays n = 20		
Control	A	B	C	A	B	C
Mean (%)	5.3	8.3	12.2	5.2	8.2	11.8
CV (%)	2.7	3.8	1.7	4.1	4.9	3.9

wavelength total Hb is measured as alkaline hematin in the same hemolisate with a cyanide-free colorimetric assay. The assay is calibrated with a synthetic glycopeptide comprising the β-N-terminal structure of HbA1c. The final result is calculated from the HbA1c/Hb ratio and expressed as a percentage of HbA1c on total Hb.

The procedure was fully automated on the Cobas Mira Plus analyzer. Blood samples anticoagulated with EDTA are directly placed on board of a Cobas Mira Plus analyzer and are automatically processed in four consecutive steps at a throughput of 36 tests per hour. The first step is hemolysis, the second is immunoassay, the third is colorimetric assay of total Hb, and last is the calculation of percent HbA1c. All reagents including calibrator and controls were ready-to-use liquids.

Diamat Method

The Diamat method is the cation-exchange HPLC system (instrument and reagents from Bio-Rad Laboratories, USA) routinely used in our laboratory.

Statistical Evaluation

The method comparison analysis has been performed using both the linear regression and the difference plot according to Bland and Altman (11). In fact, the latter technique seems to be more appropriate for giving the measure of agreement between different methods (12).

RESULTS AND DISCUSSION

The immunoturbidimetric method evaluated shows good reproducibility (Table 1). The results were obtained by analyzing 30 times three patient samples with different HbA1c concentrations and repeating the assays on the same samples daily in duplicate, in two different batches, over five days. The within-assay and between-assays imprecision (CV) was better than 4% and 5% respectively, and they were comparable with those provided by Diamat. This performance meets the analytical goal of a total reproducibility CV of 5% that is clinically recommended (13).

The method showed good linearity within the range tested (3–15%, by Diamat method), and was evaluated using different ratios of two blood samples at high and low HbA1c concentrations and assayed in duplicate in a double analytical batch (Fig.1).

No effect of the labile fraction was measured on ten specimens before and after the addition of glucose at a final concentration of 2.5 g/L followed by incubation for 4 hr at 37°C

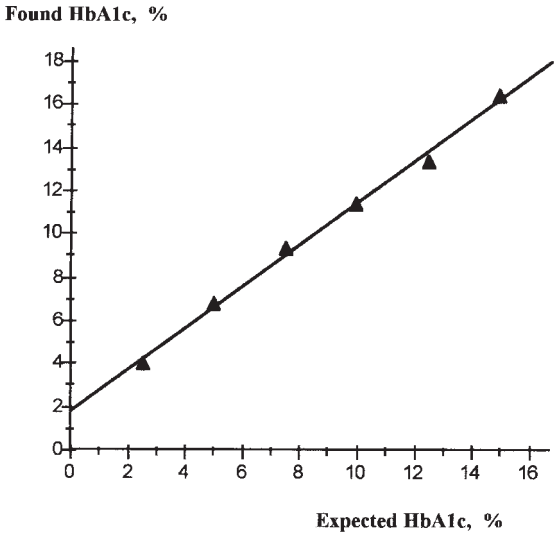

Fig. 1. Linearity of Unimate method. $y = 0.969x + 1.784\%$, $r = 0.998$, $P = 0.0001$.

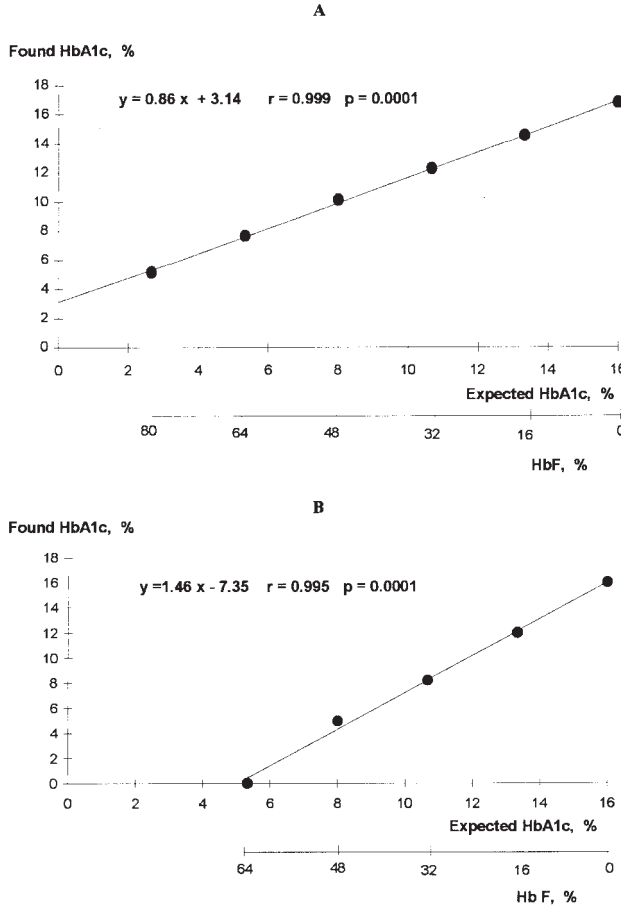

Fig. 2. Effect of fetal hemoglobin on Hba1c determination with Unimate method (**A**) and Diamat (**B**).

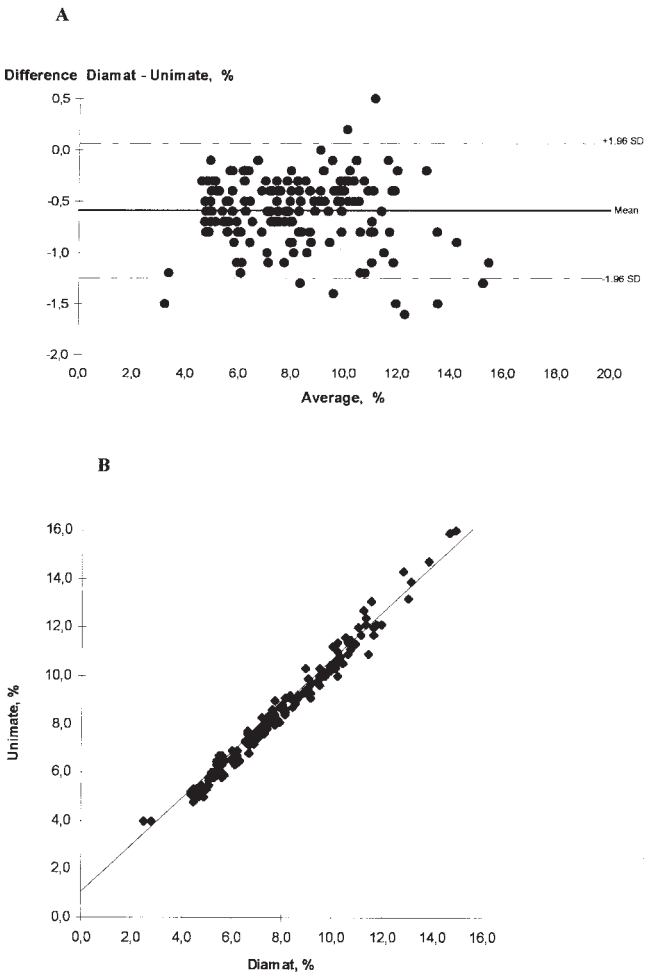

Fig. 3. Comparison between Unimate and Diamat methods. **A,** Altman and Bland plot data. Mean bias = –0.59 (95% limits = –1.25–0.06); **B,** linear regression data, Diamat (x) and Unimate (y) method: $y = 0.971\ x + 0.87\%$, $r = 0.98$, $n = 115$.

(mean difference = 0.008%, t paired data = 0.24, P = not significant).

The presence of fetal hemoglobin did not seem to affect the HbA1c determination with Unimate (Fig. 2), while the results obtained with the Diamat were inaccurate when the percentage of HbF into the sample was around 10%. Specimens were obtained by mixing blood chord with a sample collected from a poorly controlled diabetic subject in various proportions.

The Altman and Bland plot (Fig. 3A) shows a negative bias (–0.59%) for Unimate in comparison with results obtained with Diamat on 115 samples (results for samples with high HbF concentration, with documented hemoglobin variants, or from patients before dialysis were not utilized in this comparison). Conventional correlation was good ($r = 0.98$) and regression line was $y = 0.971\ x + 0.87\%$ (Fig. 3B) (results obtained by Unimate).

TABLE 2. Comparison of HbA1c Results Obtained With Unimate and Diamat on Samples Containing HbS

Hemoglobin S (% of total Hb)	HBA1c Diamat (%)	HBA1c Unimate (%)
S (30)	3.8	6.4
S (27)	3.4	6.1
S (32)	3.1	5.6
S (54)	2.5	5.6
S (38)	5.4	7.7

In samples obtained from patients with renal failure (before dialysis) a significant difference (Diamat - Unimate = (+) 0.45%) was found using the paired t test ($P = 0.009$) while in patients without renal failure the difference was negative. These results suggest that the immunoturbidimetic assay is not affected by interference of carbamylated hemoglobin, which has been reported to give falsely high results using HPLC (14). The presence of HbS does not affect the results obtained with Unimate while lower results were obtained by Diamat (Table 2).

In conclusion, the immunoturbidimetic method tested shows close agreement with the HPLC ion-exchange method and provides a highly specific and sufficiently precise assay for HbA1c. This performance makes it a suitable method (15,16).

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