Inability of Turbidimetry Method in Detecting Glycated Hemoglobin to Select Diabetes Mellitus Patients According to Their Concentrations of Blood Glucose Levels

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Background: Diabetes mellitus is a chronic illness in which there is a high blood glucose level defined as hyperglycemia, resulted by a deficiency in insulin secretion and/or in its action. Nowadays, it is being seen as a public health problem and is reaching increasing proportions with regard to the appearance of new cases. For diagnosis, sensible and accurate methods should be used to avoid complications of the sickness. The measure of glycated hemoglobin may not be used for diagnosis, but is the reference method to evaluate the grade of glycemic control in the long term, reflecting the blood glucose level in the latest 2–3 months. The aim of this study was to evaluate the grade of con-

cordance between turbidimetry and liquid chromatography methods in the glycated hemoglobin determination and to estimate the sensibility and specificity values of turbidimetry. **Methods:** This study included 133 blood samples obtained from patients and healthy donors, ageing between 18 and 80 years with glycemic values between 58 and 473 mg/dl. **Results and Conclusion:** Turbidimetry is a useful method for determining glycemic levels above 100 and over 200 mg/dl, but does not have the ability to select samples with intermediary blood glucose concentrations. J. Clin. Lab. Anal. 29:312–316, 2015. © 2014 Wiley Periodicals, Inc.

Key words: diabetes mellitus; glycated hemoglobin; LPLC; turbidimetry; glucose level

INTRODUCTION

The diabetes mellitus (DM) is a syndrome that results due to a disorder in metabolism of sugars, fats, and proteins. Nowadays, it is being seen as a public health problem and is reaching increasing proportions with regard to the appearance of new cases (1): according to the World Health Organization, approximately 150 million people have DM worldwide, and this number may double by 2025 (2). The clinical importance is due to frequent associations with numerous health problems such as vascular lesions; kidney damage; lesions in the skin, bones, and joints; injuries to nervous and cardiovascular systems; and unusual infections such as fasciitis, necrotizing myositis, and external otitis among others (2).

A high glucose concentration in the extracellular fluid (ECF) can lead to a nonenzymatic connection between

glucose and amino groups of proteins, including amino-terminal groups and lysine residues. It is termed as glycation (3). Chemically, protein glycation depends on the plasmatic level of glucose. It is practically an irreversible process in physiological concentration of hydrogen ions, thus the glucose molecule will remain on until the protein molecule is degraded. Thereby, the concentration of glycated protein is a reflex of the average level of glucose predominant in ECF during the life of that protein (3).

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Chemically, glycation is the nonenzymatic addition of a sugar residue to the amino groups of proteins. The human adult hemoglobin (Hb) usually consists of HbA (97% of the total), HbA₂ (2.5%) and HbF (0.5%). HbA is composed of four polypeptide chains, two α and two β chains (3).

The link between HbA and glucose is a continuous, slow, and irreversible type of nonenzymatic glycation. The first phase of the reaction between glucose and Hb is reversible and results in an intermediate compound termed as pre-A1C, labile or instable HbA1c, instable aldimine, or even Schiff base. The second phase results in a stable ketoamine compound, no longer dissociable, now named as HbA1c or simply A1c (4).

The International Expert Committee recommended glycated hemoglobin (HbA1c) testing as the preferred test to diagnose diabetes (5). The methods currently available for measurement of glycated hemoglobin are based on differences in ionic charge, detected either by high-performance liquid chromatography (HPLC) or microchromatography in microcolumns containing ion exchange resin (agarose gel electrophoresis), on differences in structural characteristics detected by turbidimetric immunoassay or with affinity chromatography (HPLC) using boronic acid derivatives, and finally, on chemical reactivity detected by calorimetric method with formation of 5-hydroxymethylfurfural (5HMF).

The turbidimetric method uses the antigen–antibody interaction to determine directly A1C in the whole blood (6) while low-pressure liquid chromatography (LPLC) uses low-pressure cation-exchange in conjunction with gradient elution in order to separate subtypes and variants of human hemoglobin in whole blood hemolysate (7).

This study aimed to analyze the concordance between turbidimetry and low-pressure, cation-exchange liquid chromatography in the determination of glycated hemoglobin according to blood glucose levels, and to estimate sensibility and specificity of the turbidimetric method in order to propose a new cutoff for determination of glycated hemoglobin.

PATIENTS AND METHODS

A total of 101 patients with DM treated in the outpatient specialty clinic of Faculdade de Medicina do ABC (FMABC) were randomly selected and included in this study. These patients belonged to both sexes, were aged between 18 and 80, and were DM diagnosed with more than 1 year; none of them have HIV, hepatitis B and C, neoplasias, autoimmune disease, or had been hospitalized in the last 6 months. A control group was assembled with 32 healthy blood donors, with glucose levels from 70 to 99 mg/dl, totalizing 133 samples. All patients and donors signed the free and informed consent term, which autho-

rized the use of biological samples for the study, approved by the Ethics in Research Committee (011/2009 protocol) of FMABC in São Paulo, Brazil.

To calculate the sample size, the following values for the population interclass correlation coefficient (ICC) (ρ_1) were adopted: 95% of confidence interval (CI), one-tailed, $\rho_0 = 0.85\%$, 10% of accuracy, and two replicas. A total of 10 ml of peripheral blood was obtained by venipuncture, following the collection protocol of the clinical analysis laboratory of FMABC. A total of 5 ml of blood was distributed in a tube with EDTA for determination of glycated hemoglobin by the proposed methods. In the turbidimetric assay, the agglutination complex read was made in automated spectrophotometer Flexor®-Vitalab EL200 (ELITech Clinical Systems, Holland).

In LPLC assay, the separated hemoglobin fractions were determined by light absorption at a wavelength of 415 nm. The obtained chromatogram was registered and stored by the computer within the apparatus DiaFast® (Drew Scientific Limited). The software DiaSTAT performs the analysis of the chromatogram and produces a printed report (7).

STATISTICAL ANALYSIS

In order to evaluate the concordance between both methods, the ICC was used with the respective CI of 95% (95% CI) (8), approach of Bland and Altman (9), and concordance correlation coefficient for determining precision and accuracy values (10, 11). In order to determine the sensibility and specificity of turbidimetry, the receiver operating characteristic (ROC) curve was used. The chance of finding positive and negative results among sick people by the turbidimetric method was evaluated, respectively, by positive and negative likelihood ratio values. The statistical program used was Stata 11.0.

RESULTS

A total of 101 patients with DM treated in the outpatient specialty clinic of FMABC were included in the study. Of these patients, 41.4% were male and 58.6% female. The average of age of the patients included was 58 years. The values of concordance between the studied methods (turbidimetry and chromatography) as well as the precision and accuracy are shown in Table 1.

Comparing the values of percentage of glycation of hemoglobin obtained from both methods, it was observed that the lower limit of ICC was 0.71. The precision and accuracy values from the concordance correlation coefficient were 0.79 and 0.98, respectively.

From Bland–Altman concordance limits, it is possible to see that these limits were -2.64% and 2.03% (Table 1, Fig. 1). Figure 1 shows independence between magnitude

TABLE 1. Values of Concordance Between the Methods of Turbidimetry and Liquid Chromatography in the Determination of Glycated Hemoglobin (%)

Indicators of concordance (n = 133)	Values
ICC (95% CI)	0.78 (0.71; 0.84)
r	0.79
C _b	0.98
Limits of concordance (%) (lower-upper) ^a	-2.64; 2.03

ICC, interclass correlation coefficient.
r, precision; C_b, accuracy (obtained from concordance correlation coefficient).
^aConcordance limits obtained from Bland–Altman method for the percentage of hemoglobin glycation difference (turbidimetry–liquid chromatography): (mean_{diff} – 1.96 × SD_{diff} and mean_{diff} + 1.96 × SD_{diff}).SD, standard deviation; diff, difference.

Fig. 1. Joint distribution of values of glycation of hemoglobin (%) by the methods of liquid chromatography and turbidimetry according to Bland–Altman strategy analysis (n = 133).

and dispersion of the values of percentage of glycation of hemoglobin from both methods. Figure 2 shows the line from the inclination and intercept similar to the values of the 45° line, without deviations in location and inclination.

In order to compare the methods with respect to glucose concentrations, we divided all samples (n = 133) in blood glucose levels ranges. Table 2 shows the glucose levels proposed and the number of classified samples (control and patients) according to each one. These ranges were proposed according to the interpretation of the results of glucose concentration for diagnosis of diabetes (12). From the ranges proposed in Table 2, we made the concordance between the methods of turbidimetry and LPLC.

Table 3 shows that the values of concordance between turbidimetry and LPLC are moderated only in the extreme ranges of blood glucose levels (<100 mg/dl and ≥200 mg/dL), with ICC values (95% CI) of 0.82 (0.71–0.93) and 0.70 (0.50–0.90), respectively. For levels ≥100 and <125 mg/dl and ≥125 and <200 mg/dl, the ICC values (95% CI) were low; 0.14 (0.00–0.42) and 0.55 (0.29–0.81), respectively.

Fig. 2. Joint distribution of values of glycation of hemoglobin (%) by the methods of liquid chromatography and turbidimetry according to concordance correlation coefficient (n = 133).

TABLE 2. Classification of the Samples According to Their Blood Glucose Levels

Blood glucose levels	<100 mg/dl (n = 32)	≥ 100 and <125 mg/dl (n = 48)	≥ 125 and <200 mg/dl (n = 28)	≥ 200 mg/dl (n = 25)
Male	14	16	13	1
Female	18	32	15	14

To evaluate the capacity of turbidimetry method in classifying individuals according to their concentrations of glycated hemoglobin, we compared it with the method of liquid chromatography stipulating a cutoff concentration above 6.5%. At this cutoff point, we verified that the turbidimetric method has the ability to classify 82% of the studied patients with glycated hemoglobin ≥6.5%. Still at this level, sensibility was 74.1% and specificity 87.3%. The possibility that the patient has diabetes in this cutoff is 5.85 times (Likelihood Ratio (LR) 5.85).

The capacity of turbidimetric method in comparisson with chromatographic method is show in ROC curve (Figure 3).

DISCUSSION

This comparative study showed that the turbidimetric method is indicated to measure extremes, that is, samples that present blood glucose levels below 100 and above 200 mg/dl. A satisfactory degree of concordance (0.78) was observed in the estimates of ICC and concordance between the methods of turbidimetry and liquid chromatography for the determination of glycated hemoglobin. The closer to 1 the absolute value of the correlation coefficient (r) is, the stronger will be the association; the closer to 0, the weaker it is going to be (13).

TABLE 3. Values of Concordance Between Turbidimetry and Liquid Chromatography for the Determination of Glycated Hemoglobin (%) According to Blood Glucose Levels

Indicators of concordance (<i>n</i> = 133)	<100 mg/dl (<i>n</i> = 32)	≥ 100 and <125 mg/dl (<i>n</i> = 48)	≥ 125 and <200 mg/dl (<i>n</i> = 28)	≥ 200 mg/dl (<i>n</i> = 25)
	Values			
ICC (95% CI)	0,82 (0,71; 0,93)	0,14 (0,00–0,42)	0,55 (0,29–0,81)	0,70 (0,50–0,90)
<i>r</i>	0,84	0,21	0,59	0,69
<i>C_b</i>	0,98	0,81	0,93	0,99
Limits of concordance (%) (lower-upper) ^a	–1,69; 1,25	–2,70; 1,85	–3,40; 2,56	–2,64; 2,51

ICC, interclass correlation coefficient.

r, precision; *C_b*, accuracy (obtained from concordance correlation coefficient).

^aLimits of concordance obtained from Bland–Altman method for the difference of percentage of hemoglobin glycation (turbidimetry–liquid chromatography): ($\text{mean}_{\text{diff}} - 1.96 \times \text{SD}_{\text{diff}}$ and $\text{mean}_{\text{diff}} + 1.96 \times \text{SD}_{\text{diff}}$). SD, standard deviation; diff, difference.

Fig. 3. ROC curve for the turbidimetric method compared with liquid chromatography in the classification of people according to their glycated hemoglobin.

Note: Cutoff point for liquid chromatography: >6.5%

For blood glucose levels <100 and ≥200 mg/dl, we can consider a satisfactory degree of concordance (0.82 and 0.70, respectively), evidencing that for glycemic indexes between ≥100 and <200 mg/dl, we can consider a weak association between the methods as the values of correlation coefficient are $r = 0.14$ and $r = 0.55$, respectively.

Figures 1 and 2 represent Bland–Altman graphics of concordance between LPLC and turbidimetric methods with a heterogeneous spatial distribution of plots, which indicates a relationship between the differences of the limits of concordance from –2.64 to 2.03.

The association between glycated hemoglobin (A1c) and DM has been known since the 1960s. However, the real usefulness of this laboratory parameter was recognized after the publication of two important studies: Diabetes Control and Complications Trial (DCCT) in 1993, and United Kingdom Prospective Diabetes Study (UKPDS) in 1998 (6). It was demonstrated that the

intensive control of blood glucose reduces microvascular complications, and improvements in glycemic control at all levels of achievement of HbA1c values are associated with a reduced risk of diabetes complications (7).

Ersser et al. showed that HbA1c measured in stored RBCs was associated positively linearly with mortality in patients with a confirmed diagnosis of diabetes (7). This association was independent of disease duration, use of medications, and comorbidities. Medication use and disease duration were not independently associated with the HbA1c mortality. Several randomized control trials have investigated the effect of glycemic control (HbA1c target ranging between 6.0% and 6.5%) on standard therapy (HbA1c 7.0–7.9, 1% of target) (14).

In order to compare and certify the various methods used for measurement of A1c, the National Glycohemoglobin Standardization Program (NGSP) was created in 1996 (12). Methodologies currently available for A1c dosage present high levels of accuracy and reproducibility. Nevertheless, interference factors may result in values not compatible with the real control or decontrol of diabetes. We can mention as example the limitation of the dosage of A1c in patients with hemoglobin variants (hemoglobins C, S, E, D, etc.), either in heterozygous or homozygous form. In the homozygous form, it is not possible to measure A1c due to the lack of hemoglobin A. In the heterozygous form, by its turn, some methods based on ion exchange chromatography can identify the presence of some types of hemoglobin variants, allowing a more detailed analysis of the result. It is important that the laboratory, when selecting the test method, considers the potential risk of interference and the prevalence of disease in the population group assessed (12).

Our study aimed to evaluate the ability of turbidimetry to detect glycated hemoglobin from the cutoff point established by chromatography. The crucial point in a correct interpretation of a laboratory test is its cutoff,

which means the threshold at which it becomes positive or characteristic of the disease (15). Using a cutoff point of 6.5% established by LPLC method, which is classified as reference, we found that the sensibility and specificity of turbidimetry would result in 13% of people correctly classified as altered for parameter HbA1c. Thus, about 26% of positive patients would no longer be classified for appropriate treatment. This reflects the low sensitivity and specificity by turbidimetry.

Hence, it follows that turbidimetry is a useful method to analyze blood glucose levels lower than 100 or greater than 200 mg/dl, but does not have the ability to select individuals with intermediate concentrations and classification for diabetes.

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