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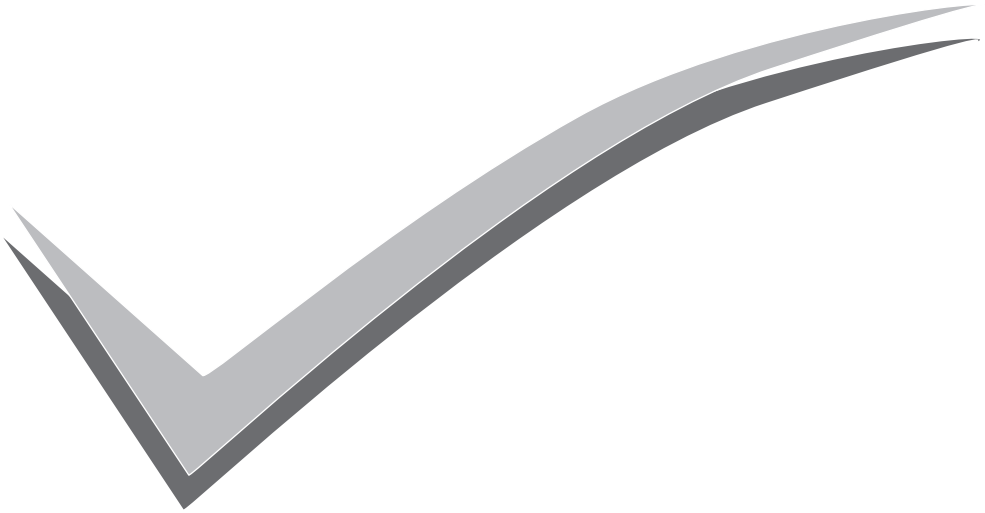

QUANTIA® HBA1c

Quantitative Turbidimetric Immunoassay for HBA1c

Tulip Group

Performance Evaluations

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SCIENTIFIC REPORT

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1.	Underestimation of HbA1c values by the rapid boronate affinity assay as compared to HPLC, the gold standard and a direct immunoturbidimetric assay for assessment of glycemic control in diabetics.

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Underestimation of HbA1c values by the rapid boronate affinity assay as compared to HPLC, the gold standard and a direct immunoturbidimetric assay for assessment of glycemic control in diabetics.

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Abstract

Objective - To assess the accuracy of HbA1c values obtained with a rapid boronate affinity assay and a direct immunoturbidimetric assay, using HPLC (ion exchange) as the gold standard, for assessment of blood glucose control in diabetics performed in clinical laboratories.

Study Design and Methods

Specimens from 25 known diabetic patients visiting a reputed laboratory for HbA1c assessment were used in the study. The specimens were analyzed with the rapid boronate affinity method and the direct immunoturbidimetric method for HbA1c in two different laboratories. The results of both the methods were compared with the results obtained by the gold standard HPLC method (Biorad D-10) for their accuracy in classification of patients with glycemic control, based on ADA recommendations. Accuracy was determined by estimating the percentage variation, and difference in HbA1c values obtained with respect to the reference method for each specimen. Passing and Bablok regression and correlation for each method was calculated using HPLC as the reference method.

Results

Of the 17 patients with HbA1c > 8% (inadequate glycemic control) by HPLC, wherein intervention is recommended by ADA, the rapid boronate affinity assay misclassified 8 (47%) patients as having adequate glycemic control. The immunoturbidimetric assay classified all the 17 patients correctly. The range of HbA1c values with HPLC was from 7.3 -11.5%. In specimens with inadequate control (classified by HPLC), the boronate affinity assay underestimated HbA1c values by 19% in one specimen and between 11-17.5% in 7 specimens. Whereas none of the specimens were underestimated for HbA1c by the immunoturbidimetric assay in this group.

The boronate affinity assay exhibited a variation between 10 – 19.3 % in 16 specimens with HbA1c values ranging between 7.3 -11.4 % by HPLC, of which 5 specimens had HbA1c values between 7-8%. The immunoturbidimetric assay demonstrated an acceptable variation of < 5% in 17 samples and >10 % in none of the samples used in evaluation (refer table 3A).

The correlation coefficient for immunoturbidimetric assay with HPLC [r=0.98 (P< 0.0001)], was much better than that of boronate affinity assay with HPLC [r= 0.94 (p<0.0001)].

Conclusions

The boronate affinity assay underestimated HbA1c values in 47% patients, who had inadequate blood glucose control, misclassifying them as having adequate control. Underestimation of HbA1c values may put patients with inadequate control, at risk of developing diabetic complications, by denying the required change in therapy or lifestyle modifications. The immunoturbidimetric assay demonstrated good correlation with the reference method and better accuracy in classifying patients with inadequate control. The use of the simple direct immunoturbidimetric assay, that is adaptable on most semi automated analyzers available in routine laboratories, would definitely be a boon for accurate measurement of HbA1c in our country.

Abbreviations: DCCT, Diabetes Control and Complications Trial; ADA, American Diabetes Association; HPLC, High Performance Liquid Chromatography; POCT Point Of Care Test

Background

Monitoring of glycemic status, as performed by patients and health care providers, is considered a cornerstone in diabetes care. The results of monitoring are used to assess the efficacy of therapy and to guide adjustments in medical nutrition therapy, exercise, and medications to achieve the best possible glucose control.

The glycosylated hemoglobin assay (GHb) is a powerful research tool that is unique as a retrospective index of glucose control over time in patient with diabetes [6].

The traditional methods of assessing blood glucose control appear to be both non-specific and insensitive with regard to control over time. Since the assessment of day to day metabolic control remains important in the management of diabetes, indexes of short term control, such as measurements of glycosuria, remain important. More direct and accurate methods, such as blood glucose home monitoring, are currently recommended to aid in the day to day management of diabetes. Although refinement of the indexes of short term glucose control may lead to better appreciation of long term control, no currently available single assessment is as informative about long-term control as the glycosylated hemoglobin assay. Regular measurement of Hemoglobin A1c leads to changes in diabetes treatment and improvement of metabolic control, indicated by a lowering of HbA1c values[9]. It is therefore imperative that the classification of a patient's glycemic control by using this measurement be both accurate and reproducible .

The term glycosylated hemoglobin encompasses both Hemoglobin A1 (HbA1) and Hemoglobin A1c (HbA1c). HbA1 refers to the non-enzymatic binding of several species of carbohydrate to hemoglobin, whereas in HbA1c the carbohydrate is specifically glucose [1]. More recently HbA1c is defined as Hb that is irreversibly glycated at one or both N-terminal valines of the β-chain [2].

A major difficulty associated with GHb determination is the variable and unstandardized methodology, often measuring different chemical moieties of glycated hemoglobin(s), thereby producing irreproducible and incomparable results[5]. The procedures applying boronate affinity principle measure total GHb, a value which is not interconvertible with hemoglobin A1c (HbA1c) and is therefore questionable in terms of clinical comparison[5]. Errors in HbA1c results are amplified when the result is related to a blood glucose value: each 1 percent error in the measurement of HbA1c leads to an error of approximately 35 mg/dl in the blood glucose level [7].

Kendra L Schwartz et al[3] in a study compared the results of Micromat II (boronate affinity) with other laboratory methods the Primus model 386 (boronate affinity-HPLC), Roche integra 800 (immunoturbidimetry) and the Tosoh A1c 2.2 plus (Ion exchange HPLC). The mean HbA1c values obtained from Micromat II was significantly lower than that yielded from the three types of laboratory analysis and this difference spanned the treatment threshold level currently recommended by ADA. For some patients the Micromat II rapid test yielded a test result that was below the ADA threshold of 7%, while the other laboratory methods produced a test result above 7%, suggesting the need for intensive therapy.

R C Hawkins[4] assessed the analytical inaccuracy by analysis of 110 samples on different HbA1c analytical platforms Biorad Diastat, Drew DS5, Bayer DCA 2000, Nycomed Nycocard and Roche Tinaquant. Analytical imprecision was assessed by analysis of two levels of patient samples run twice in the morning and afternoon daily for six days, as well as analysis of two levels of commercial controls. Within-run, between run, between day and total imprecision were calculated. The Tinaquant system was most precise with CVs of less than 3% using patient samples. The DS5 and Nycocard systems were less precise with CVs of 5.1-8.6% with patient samples.

In our country (India), most of the routine laboratories use rapid boronate affinity assays (Nycocard, Micromat II) for HbA1c considering factors such as cost, speed of analysis and ease of use. Whereas the referral laboratories use automated ion-exchange HPLC methods such as Tosoh A1c 2.2, Biorad D-10, Diamat and Diastat systems and a few use automated immunoturbidimetry methods which include Roche Tinaquant II and Unimate for achieving specific and accurate HbA1c measurements.

To suffice the increasing demand of HbA1c measurement producing a clinically valuable results in routine clinical laboratories, a simple, specific procedure for measurement of HbA1c is needed. The recently developed immunoturbidimetric assay provides a unique advantage by combining specificity of an immunoassay and adaptability to spectrophotometers, routinely used in laboratories as chemistry analyzers.

The increasing use of HbA1c in the monitoring of diabetic control and recognition of significant interlaboratory variability, prompted us to investigate the accuracy of the widely used rapid POCT test based on boronate affinity and the new immunoturbidimetric assay that is adaptable on semi automated analyzers available in routine clinical laboratories.

We therefore designed this study to ascertain the accuracy of HbA1c measurements with the widely used rapid boronate affinity method that measures GHb but reports HbA1c and the direct agglutination method (immunoturbidimetric assay) which directly measures HbA1c for classifying glycemic control in diabetics in comparison to the gold standard ion exchange HPLC method, traceable to DCCT.

Subjects and Methods

Diabetic patient samples were categorized based on ADA recommended guidelines. The ADA recommends that the goal of therapy should be an A1C result of < 7% and that physicians should evaluate and, in most cases, significantly change the treatment regimen in patients with A1c test results consistently > 8%. For convenience, in our study, we classified the patients with HbA1c > 8% as inadequate control and HbA1c between 7-8% as adequate control.

HbA1c values of known diabetic patients were analyzed by the ion exchange HPLC method (used as a reference method in this study) in a reputed laboratory[10]. Specimens with > 7% HbA1c were identified and divided into two aliquots. One aliquot was sent to another laboratory[10] for testing with the rapid boronate affinity assay. The second aliquot was sent to a different laboratory[10] for estimating HbA1c values with the immunoturbidimetric assay [Quantia HbA1c Mfd. by Tulip Diagnostics (P) Ltd.]. The results of both methods were compared with the results obtained by the HPLC method for determining their accuracy in classification of patients with glycemic control. Accuracy was determined by estimating the percentage variation, and difference in HbA1c values obtained with respect to the reference method for each specimen. Passing and Bablok regression and correlation for both the method was calculated using medcalc software with HPLC as the reference method.

Assay Methods and Principle

Boronate affinity method

The kit uses test devices with a porous membrane filter, test tubes prefilled with reagent and washing solution. The reagent contains agents that lyse erythrocytes and precipitate hemoglobin specifically, as well as a blue boronic acid conjugate that binds cis -diol of glycated hemoglobin. When blood is added to the reagent, the erythrocytes immediately lyse. All hemoglobin precipitates. The boronic acid conjugate binds to Cis-diol of glycated hemoglobin. The mixture is added to the test device, and all the conjugate bound and unbound precipitated hemoglobin remains on top of the filter. The precipitate is analysed by measuring the blue (glycated hemoglobin) and red (non glycated hemoglobin) color intensity with a reader based on spectral reflectance. The ratio between the two is calculated and the result is converted to HbA1c.

Immunoturbidimetric assay

The immunoturbidimetric assay for direct determination of HbA1c is based on the principle of agglutination reaction. The test specimen after treatment with Hemolysing solution is allowed to react with latex reagent (R1). Total Hb and HbA1c bind with same affinity to latex particles. The amount of binding is proportional to the relative concentration of both substances in blood. The reaction mixture is then allowed to react with mouse anti-human HbA1c monoclonal antibody reagent (R2) wherein the mouse anti-human HbA1c antibody binds to the HbA1c on the latex. Goat anti-mouse human IgG reagent (R3) is then allowed to interact with the above reaction mixture which interacts with the HbA1c-mouse anti-human HbA1c complex resulting in agglutination reaction that is measured at 630 nm. The increase in turbidity directly corresponds to the concentration of HbA1c in the test specimen. Separate measurement of total Hb is not required in this method.

A calibration curve is prepared using three level HbA1c calibrators traceable to the DCCT. The % HbA1c values for the test specimen are then interpolated from the calibration curve.

Ion exchange HPLC

The D - 10 Hemoglobin program utilizes principles of ion exchange high performance liquid chromatography (HPLC). The samples are automatically diluted on the D - 10 and injected into the analytical cartridge. The D -10 delivers a programmed buffer gradient of increasing ionic strength to the cartridge, where the hemoglobin's are separated based on their ionic interactions with the cartridge material. The separated hemoglobin's then pass through the flow cell of the filter photometer, where changes in the absorbance at 415 nm are measured. The D -10 software performs reduction of raw data collected from each analysis. Two level calibration is used for quantitation of the HbA1c values. A sample report and a chromatogram are generated for each sample. The A1c area is calculated using an exponentially modified Gaussian (EMG) algorithm that excludes the labile HbA1c and carbamylated peak areas from the A1c peak area.

Results

Table 1: Comparison of HbA1c values obtained for 25 specimens with boronate affinity and immunoturbidimetric assays with HPLC.

Sr. No	Sample ID	% HbA1c values			Boronate affinity		Immunoturbidimetry	
		Boronate Affinity	HPLC	Immuno-turbidimetry	HbA1c value difference from HPLC	% Variation	HbA1c value ifference from HPLC	% Variation
1	278	8.8	10.9	11.5	2.1	19.3	0.6	5.5
2	584	7.8	9.3	10	1.5	16.1	0.7	7.5
3	530	7.2	8.2	8.7	1	12.2	0.5	6.1
4	585	6.9	7.3	7.9	0.4	5.5	0.6	8.2
5	380	9.4	11.4	12.3	2	17.5	0.9	7.9
6	583	6.7	7.6	8.2	0.9	11.8	0.6	7.9
7	623	7.8	9.2	9.7	1.4	15.2	0.5	5.4
8	648	6.8	7.7	7.8	0.9	11.7	0.1	1.3
9	727	7	7.9	7.7	0.9	11.4	0.2	2.5
10	728	9.1	9.9	10.8	0.8	8.1	0.9	9.1
11	764	7.1	8.1	8.3	1	12.3	0.2	2.5
12	797	8.4	9.3	9.6	0.9	9.7	0.3	3.2
13	903	8.3	8.9	8.9	0.6	6.7	0	0.0
14	904	7.3	8	8.3	0.7	8.8	0.3	3.8
15	264	6.4	7.7	7.6	1.3	16.9	0.1	1.3
16	009	7	8.1	8.2	1.1	13.6	0.1	1.2
17	229	6.7	7.5	7.8	0.8	10.7	0.3	4.0
18	103	9.8	11.3	11.4	1.5	13.3	0.1	0.9
19	273	7.5	8.4	8.7	0.9	10.7	0.3	3.6
20	290	8.8	9.7	9.8	0.9	9.3	0.1	1.0
21	306	7.3	8.2	8.6	0.9	11.0	0.4	4.9
22	628	6.9	7.4	7.7	0.5	6.8	0.3	4.1
23	896	10.3	10.7	11	0.4	3.7	0.3	2.8
24	805	8.6	9.5	9.9	0.9	9.5	0.4	4.2
25	038	7.3	8.3	8.5	1	12.0	0.2	2.4
Mean		7.81	8.82	9.16	1.01	11.35	0.34	3.75

Table 2: Classification of patients for glycemic control with rapid boronate affinity and immunoturbidimetric assays, considering classification by HPLC as reference.

No. of patients classified for glycemic control by HPLC	No of patients classified for glycemic control		No of patients misclassified for glycemic control	
	Boronate affinity	Immunoturbidimetry	Boronate affinity	Immunoturbidimetry
HbA1c < 7% (therapeutic goal) n*= 0	6	0	6↓	0
HbA1c 7-8% (adequate control) n* = 8	10	6	2↓	2↑
HbA1c > 8% (inadequate control) n* = 17	9	19	8↓	2↑

↓ underestimation, ↑ overestimation, n = No. of Patients

The range of HbA1c values with HPLC was from 7.3 -11.4%. The mean HbA1c value with boronate affinity method was 7.81%, which was significantly lower to the HPLC mean of 8.82%.The mean HbA1c with immunoturbidimetric assay was 9.16% closer to the HPLC mean (refer Table 1).

Of the 17 patients with HbA1c > 8% by HPLC, wherein intervention is suggested, the rapid boronate affinity method classified only 9 patients (53%) correctly, misclassifying the remaining 8 patients (47%) as having adequate glycemic control, whereas the immunoturbidimetric assay classified all the 17 patients correctly (refer table 2 and 5).

In specimens with inadequate control (classified by HPLC), the boronate affinity assay underestimated HbA1c values by 19% in one specimen and between 11-17.5% in 7 specimens. Whereas none of the specimens were underestimated for HbA1c by the immunoturbidimetric assay in this group.

The boronate affinity assay exhibited a variation between 10 – 19.3 % in 16 specimens with HbA1c value ranging between 7.3 -11.4 % by HPLC, of which 5 specimens had HbA1c values between 7-8%.The immunoturbidimetric assay demonstrated an acceptable variation of < 5% in 16 samples and >10 % in none of the samples used in the evaluation (refer table 3A).

The immunoturbidimetric assay demonstrated a much better correlation (r = 0.98, p < 0.0001) compared to Boronate affinity assay (r = 0.94) with the reference method (HPLC).

Table 3 A: Underestimation of HbA1c values measured by boronate affinity and immunoturbidimetric assay as compared to HPLC

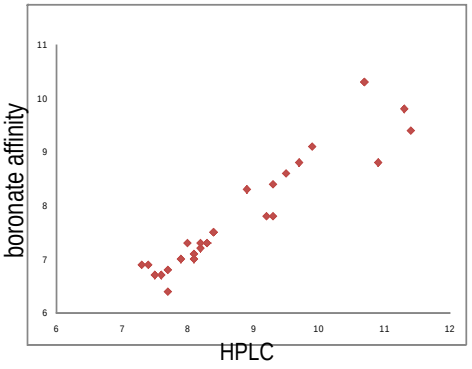
Method	No. of specimens with underestimated HbA1c value	
	1-2 % HbA1c	>2% HbA1c
Boronate affinity assay	8	1
Immunoturbidimetric assay	0	0

Table 3B: Variation of boronate affinity and immunoturbidimetric assay from HPLC

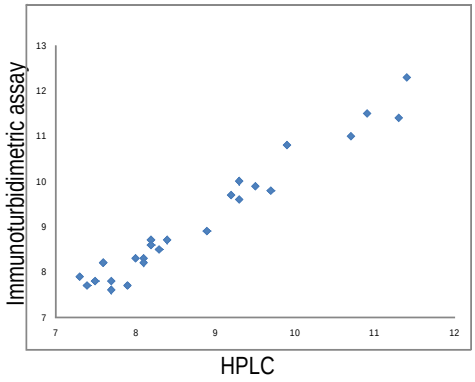
Method	No of specimens with % variation compared to HPLC		
	< 5%	5 -10%	>10%
Boronate affinity assay	1	8	16
Immunoturbidimetric assay	17	8	0

Table 4: Correlation coefficient and Passing and Bablok Regression

Method	Passing and Bablok regression			Correlation r (95% CI)
	y	Slope (95% CI)	Intercept (95% CI)	
Immunoturbidimetry	TIA	1.08 (1.00 – 1.21)	-0.44 (-1.50 - 0.30)	0.98 (0.96 - 0.99)
Boronate affinity	BA	0.88 (0.72–1.00)	0.03 (-0.90 - 1.28)	0.94 (0.87 - 0.97)



Correlation of boronate affinity assay with HPLC



Correlation of Immunoturbidimetric assay with HPLC

Table 5: Clinical Impact of HbA1c values

Reference method (HPLC)	Immunoturbidimetry	Boronate Affinity
Total No. of patients Needing intervention (HbA1c > 8%) n*= 17	No. of Patients needing intervention (HbA1c > 8%) 19	No. of Patients needing intervention (HbA1c > 8%) 9
	No. of Patients denied intervention due to underestimation 0	No. of Patients denied intervention due to underestimation 8

Discussion

The availability of POCT for HbA1c is developing increasing interest in both clinicians and laboratories. Although factors such as cost ease of use, speed of analysis and blood volume should be considered when choosing a suitable system, accuracy of results is imperative for monitoring glycemic control when patients frequently change laboratories for determining HbA1c. Clinicians must be aware that results reported on the same sample can vary substantially, depending on the glycated Hb moiety measured and the method used. With several HbA1c methods available, it is important to ensure that all HbA1c results used for patient management are reliable and comparable between laboratories. The quality of results must not be sacrificed for convenience. The rapid boronate affinity assay in this study demonstrated significantly lower HbA1c values compared to ion exchange HPLC an observation similar to the study by Kendra L Schwartz et al. The boron affinity assay underestimated HbA1c values in 47% patients with inadequate glycemic control, misclassifying them as adequate glycemic control , that would result in a denial of intervention to these patients.

Underestimation of HbA1c values may put patients with inadequate glycemic control, at risk of developing diabetic complications, by denying the required change in therapy or lifestyle modifications. In relation to the diabetes control and complications trial (DCCT) this inaccuracy may have considerable consequences for the long term well being of diabetic patients and may also influence the allocation of resources towards their treatment.

The direct immunoturbidimetric assay in this study demonstrated better accuracy and excellent correlation with the reference method in classifying patients with inadequate control. The overestimation of HbA1c values observed in 2 specimens (refer table 2) in the adequate control group, with the immunoturbidimetric assay will not have any adverse outcome, but facilitate glycemic control suggesting intervention to achieve < 7% HbA1c (ADA goal), benefitting the patient. The study by M. Vucic et al. also established that the immunoturbidimetric assay produced results that were highly comparable with ion-exchange chromatography (Mono S Column) in a wide range of HbA1c values (n=117, r= 0.989) [5].

The lower values obtained with the rapid assay based on boronate affinity method can be attributed to several factors; a) use of inbuilt calibration which does not take into account laboratory conditions and instrument to instrument variations, b) Indirect measurement of HbA1c by using measurement of GHb and total Hb c) Use of small sample volume (5µ l) that is prone to error for lysate preparation d) For optimum results the reagents and devices needs to be brought to 20-25^{0}C before testing. It is practically difficult to control the prevailing temperature between 20-25^{0}C in laboratories/clinics across the country all year around.

On the other hand immunoturbidimetric assay (Quantia HbA1c) : a) Recommends calibration with calibrators traceable to the DCCT, taking into account laboratory conditions, instrument variations and operator techniques. b) Provides direct measurement of HbA1c without measuring total Hb c) Utilizes 10 µl sample for lysate preparation thereby reducing the possibility of error d) Works optimally at 37^{0}C, a temperature that is available in most analyzers and therefore can be easily controlled in laboratory conditions.

Is it advisable to use a method that would compromise accuracy for convenience? In conclusion, clinicians should be aware that rapid HbA1c technology may produce results that are lower than the gold standard ion exchange HPLC suggesting a different treatment strategy.

This study, though it uses a small sample size for evaluation, does indicate the need for adopting a suitable HbA1c method that would allow correct classification of patients with diabetic control aligned to the DCCT. However as the sample size was small, more studies need to be done in this context to ensure inter comparability of HbA1c results between laboratories to achieve efficacy in monitoring glycemic control in laboratories within the country. The use of the simple direct immunoturbidimetric assay that can be measured on semi automated chemistry analyzers would definitely become a boon for accurate HbA1c measurement in monitoring glycemic control in our country.

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