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Evaluation of an Immunoturbidimetric Assay for Haemoglobin A_{1c} on a Cobas® Mira S Analyser

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Summary: We evaluated a homogenous immunoturbidimetric assay for haemoglobin A_{1c} (Tina-quant® Haemoglobin A_{1c}, Boehringer Mannheim, GmbH, Mannheim, Germany) adapted to a Cobas® Mira S analyser (F. Hoffmann-La Roche & Co., Basel, Switzerland) and not requiring sample pretreatment. Between-day CV's determined over a 6 week period were 5.9% and 5.3% for mean haemoglobin A_{1c} values of 5.4% and 17.0% of total haemoglobin respectively. The imprecision was higher than with an in-house ion-exchange high performance liquid chromatographic method. Bilirubin, triacylglycerols and the labile fraction of haemoglobin A_{1c} did not interfere with the assay of haemoglobin A_{1c}. Fetal haemoglobin is not recognized by the antibodies used. Results correlated well with those obtained by high performance liquid chromatography. In conclusion, the Tina-quant® assay is not prone to common interferences and allows the rapid and automated determination of haemoglobin A_{1c} on open photometric analysers.

Introduction

Glycohaemoglobin measurement is widely used to monitor glycaemic control in diabetic patients (1–4). Glycated haemoglobin comprises a variety of molecular species formed by non-enzymatic glycation of haemoglobin (Hb) components at various sites. The reaction of glucose with the N-terminal amino-group of the haemoglobin β-chain generates haemoglobin A_{1c} (HbA_{1c}); the latter fraction is best correlated with the average blood glucose concentration (1).

Haemoglobin A_{1c} values provide a time-integrated retrospective view on circulating glucose levels over a 4–6 week period. Repeated determinations of HbA_{1c} in diabetic patients help to assess the compliance with therapy and the risk to develop the devastating chronic complications of the disease (1). Many widely used methods for quantitation of HbA_{1c}, such as ion-exchange microcolumn methods, high performance liquid chromatography (HPLC) and electrophoretic techniques, exploit the charge differences between glycated and non-glycated Hb. Boronate affinity chromatography is based on the structural characteristics of the carbohydrate moiety (1).

HPLC-methods measure HbA_{1c} with great precision, but are labour intensive and have a rather low throughput (1). New automated methods have recently been developed for the fast and reliable determination of HbA_{1c} (5–13). In this study, we evaluated a new homogenous immunoassay (9–11) for the determination of HbA_{1c} (Tina-quant® Haemoglobin A_{1c}, Boehringer Mannheim GmbH, Mannheim, Germany) adapted to a Cobas® MiraS analyser (Hoffmann-La Roche & Co Ltd, Basel, Switzerland).

Materials and Methods

Blood samples

Whole-blood specimens were collected into Monovette-tubes coated with lithium heparin anticoagulant (Sarstedt, Essen, Belgium). Samples were stored at 2–8 °C and analysed within one week after collection.

Assay procedure

Haemolysates were prepared by adding 10 µl of whole blood to 1 ml cyanide-free haemolysis reagent. No pretreatment for removal of the labile fraction was performed. Total Hb and HbA_{1c} were measured on a Cobas® Mira S analyser operated at 37 °C, according to the manufacturers' instructions. The test conditions are listed in table 1. The instrument calculates the HbA_{1c}/total Hb concentration ratio and expresses HbA_{1c} results as fraction of total Hb.

Comparison method: high performance liquid chromatography

The HPLC procedure consisted in an adaptation of the method of *Stenman* et al. (14). Blood cells were sedimented by centrifugation at 1000 *g* for 10 minutes at room temperature. The packed cells were washed three times with a 9 g/l NaCl solution. One volume of packed washed cells was lysed by addition of one volume of water. Delipidation was performed by mixing five volumes of lysate with two volumes of carbon tetrachloride. The mixture was centrifuged for 5 minutes at room temperature and 100 µl of the clear aqueous phase was diluted with 3 ml of hydrolysis buffer (25 mmol/l phosphate buffer pH 4.85 with 4 mmol/l KCN). After a 3 h incubation at 37 °C to remove the labile glycated fraction, a 20 µl aliquot of the diluted haemolysate was injected onto a Mono S HR 5/5 column (Pharmacia LKB Biotechnology, AB, Uppsala, Sweden). The HPLC method was validated by repeated participation in an international external quality control program (Stichting Kwaliteitsbewaking voor Klinsch-Chemische Ziekenhuislaboratoria, the Netherlands).

Tab. 1 Adaptation of Tina-quant® HbA_{1c} to Cobas® Mira S.

Conditions	HbA_{1c}	Total Hb
Measurement mode	absorbance	absorbance
Calibration		
– mode	logit/log5	linear
– calibrators (reagent 3)		
n	5	1
duplicates	yes	yes
units	g/l	g/l
range	0.0–25.1	133[f]
Reagent blank	no	yes
Wavelength (nm)	340	600
Cycle 1:	8 µl sample	230 µl reagent 4[a]
– pipetting	15 µl H_2O	
	350 µl reagent 1[b]	
– reading	–	absorbance at t = 25s
Cycle 2:	–	30 µl sample
– pipetting	–	20 µl H_2O
Cycle 4:		
– reading	–	absorbance at t = 100s
Cycle 12:		
– reading	absorbance at t = 5 min	–
Cycle 13:	70 µl reagent 2[c]	–
– pipetting	20 µl H_2O	–
Cycle 30:		
– reading	absorbance at t = 12.5 min	–

[a] reagent 4: 0.2 mol/l phosphate buffer (pH 7.4)
[b] reagent 1: sheep anti HbA_{1c} ≥ 0.5 g/l in 0.05 mol/l N-morpholino-ethanesulphonic acid (MES), pH 6.2
[c] reagent 2: haemoglobin A_{1c} polyhapten ≥ 20 mg/l in 0.05 MES buffer, pH 6.2

Imprecision studies

Between-day imprecision was assessed by analysing erythrolysates (stored at −70 °C and routinely used as control material for the in-house HPLC) over a 6 week period using 5 calibrations and two different test pack lots. The same erythrolysates were also analysed with the HPLC method over the same period.

Linearity studies

Linearity was evaluated by preparing mixtures of two haemolysates with equal total Hb concentration (130 g/l), one from a patient with poorly controlled diabetes (14.5% HbA_{1c}) and one from a non-diabetic subject (4.0% HbA_{1c}). The mixtures were analysed in duplicate in the same analytical run.

Interference studies

To evaluate the effect of the total Hb concentration, packed cells were prepared from 5 ml of an anticoagulated blood sample (123 g/l total Hb, 5.6% HbA_{1c}) and 100 µl aliquots of the cell suspension were mixed with various volumes (50–400 µl) of the original plasma. In this way, samples were prepared with a fixed HbA_{1c} fraction and a total Hb concentration varying between 66 and 200 g/l.

Interference by the labile HbA_{1c} fraction was evaluated by supplementing an anticoagulated whole blood sample with 50 mmol/l *D*-glucose. Haemolysates were analysed before and after 2 h and 4 h incubation at 37 °C. The formation of the labile fraction was confirmed by analysing the samples with HPLC without removal of the labile fraction, i.e. with omission of the 3 h incubation in acid hydrolysis buffer.

Bilirubin interference was evaluated by preparing bilirubin supplemented samples according to the procedure of *Glick* et al. (15). Two different experiments were performed to evaluate the effect of lipaemia. In the first experiment, a whole blood sample was supplemented with Intralipid® 20% (KabiVitrum Inc., Alameda, CA 94501) up to 1 g/l. In the second experiment, haemolysates were supplemented with VLDL-rich plasma to test the effect of endogenous lipids (and other endogenous compounds).

The effect of foetal haemoglobin (HbF) was checked as follows: cord blood (70% HbF as determined by HPLC) was mixed with adult blood (7.5% HbA_{1c} as determined by immunoassay) in various proportions (volume fractions of HbF = 0.2, 0.4, 0.6 and 0.8). The total Hb concentration was 134 g/l in both samples as determined by the Tina-quant® total Hb assay. The original samples and the mixtures were analysed by immunoassay and the results (expressed in %) compared with the expected values, calculated as 7.5 (1-volume fraction of cord blood).

Results

Imprecision studies

Between-day imprecision was assessed by analysing erythrolysates over a 6 week period (n = 12) and amounted to 6.9% and 5.3% for HbA_{1c} values of 5.4% and 17.0% respectively. For comparison, between-day CV's with our in-house HPLC-method were only 1.7% and 0.8% for the same erythrolysates analysed over the same period (n = 12). Over a 9 month period (n = 12), CV's with HPLC were 1.8% and 0.8%.

Linearity studies

Assay linearity was confirmed between 4.0% and 14.5% HbA_{1c} by analysing mixtures of samples with low and high HbA_{1c} content. The correlation between measured (y) and calculated (x) values was: $y = 0.99x - 0.06$ ($r = 0.9993$; $s_{yx} = 0.16\%$).

Interference studies

Five samples with total Hb ranging from 66 g/l to 200 g/l, were prepared by mixing packed red cells from a

non-diabetic subject with various volumes of the original plasma. The fraction of HbA_{1c} as determined with the Tina-quant® method averaged 5.5% (S.D. = 0.3%; range = 4.9%–5.8%) and was not correlated with the total Hb concentration (p = 0.25).

Possible interference by the labile fraction of HbA_{1c} was evaluated by incubation of an anticoagulated blood sample with 50 mmol/l glucose at 37 °C. Under these conditions, large amounts of labile fraction were formed as evidenced by the HPLC measurements performed without removal of the labile fraction (see methods): the apparent HbA_{1c} (i.e. HbA_{1c} plus labile fraction) rose from 6.7% before incubation to 16.2% and 23.4% after 2 h and 4 h of incubation respectively. In contrast, HbA_{1c} determined by immunoassay ranged between 5.1 and 5.7%, reflecting method imprecision rather than interference by the labile fraction.

Bilirubin up to 350 µmol/l (20 mg/dl) did not change the results of the immunoassay by more than 0.5% at a level of 6.1% HbA_{1c}.

An important negative interference was observed in Intralipid® supplemented samples. Haemoglobin A_{1c} values were 6.2%, 4.8%, 4.4%, 4.0% and 3.5% for samples supplemented with Intralipid® equivalent to final triacylglycerols concentrations of 0.0 g/l, 0.25 g/l, 0.50 g/l, 0.75 g/l and 1.0 g/l respectively. Very low density lipoproteins (VLDL) and other endogenous compounds, however, did not interfere in the immunosassay as assessed by supplementing erythrolysates with VLDL-rich plasma. Haemoglobin A_{1c} averaged 9.9% (S.D. = 0.5%; range 8.9–10.4%) in haemolysates prepared from washed reticulocytes and spiked with different volumes of VLDL-rich plasma (n = 6; up to 50 µl plasma per ml haemolysate). The correlation between HbA_{1c} values and the added plasma volume was not statistically significant (p = 0.15).

Haemoglobin A_{1c} was not detected (< 0.5%) in cord blood samples, indicating that HbF, whether glycated or acetylated, is not recognized by the antibodies used in the Tina-quant® immunoassay. Foetal haemoglobin also did not affect the measurement of HbA_{1c}, as judged by the linear relation between measured (y) and expected (x) values for the mixtures of cord blood and adult blood (y = 0.75 + 0.92x; r = 0.995).

Method comparison

Haemoglobin A_{1c} values obtained with the immunoassay correlated well with those obtained by the HPLC method, albeit with a significant slope and intercept effect (fig. 1). With the exception of two samples (one with 40% HbC and one with 35% HbS), all samples containing increased amounts of variant Hb (2–20% HbF, 22–37% HbS, 39% HbC and 37% HbD) closely followed the correlation for HbA containing samples (fig. 2).

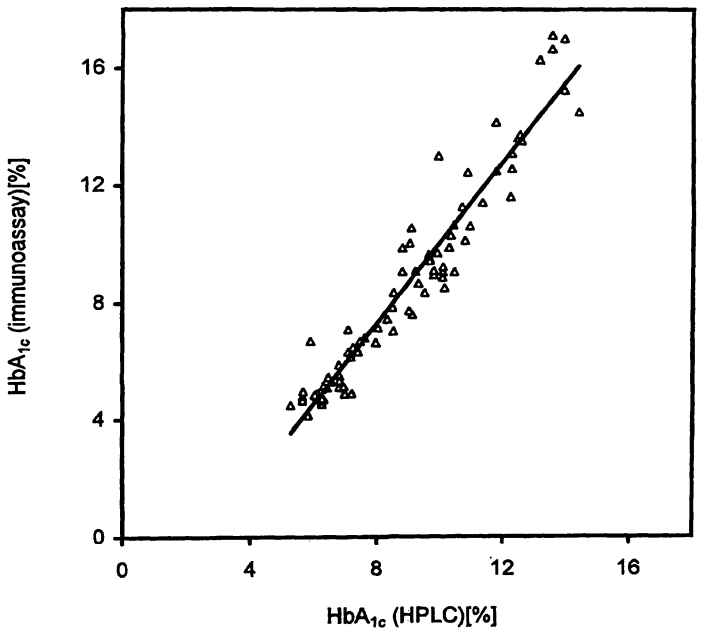

Fig. 1 Correlation of HbA_{1c} results obtained with the Tina-quant® immunoassay with results obtained by HPLC for 85 adult patients without genetic Hb variants.
Regression line by linear regression analysis: y = 1.37x − 3.72; r = 0.96; S_{yx} = 0.93%. The slope was significantly different from 1 (95% confidence interval = 1.29–1.46) and the intercept was significantly different from 0 [95% confidence interval = (−4.50)– (−2.94)].

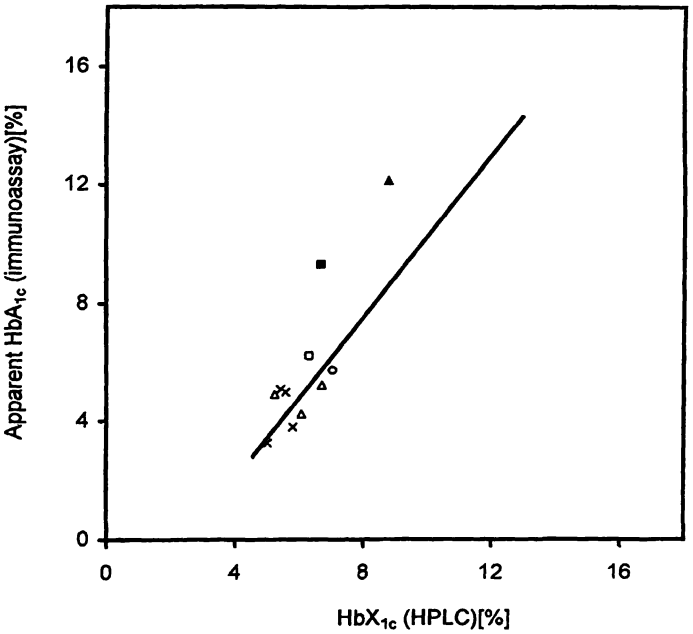

Fig. 2 Correlation of HbA_{1c} results obtained with the Tina-quant® immunoassay with results obtained by HPLC for 11 patients with significant amounts of variant Hb [HbS (△, ▲); HbC (□, ■); HbD (○); HbF (x)].
HbX_{1c} represents the sum of % HbA_{1c} and % HbC_{1c}, HbS_{1c} or HbD_{1c}; HbC_{1c} is measured; HbS_{1c} is computed according to the formula: % HbS_{1c} = % HbS x (% HbA_{1c}/% HbA); HbD_{1c} was calculated similarly, taking into account the % HbD instead of the % HbS.
The solid line represents the regression line from figure 1; with the exception of two samples (■ with 40% HbC and ▲ with 35% HbS) all samples containing increased amounts of variant Hb followed the correlation for samples containing almost exclusively HbA.

Discussion

The present immunoturbidimetric assay allows the fast and automated determination of HbA_{1c} on open photometric analysers (9–11). In contrast to the more labour-intensive HPLC method, this assay requires no extensive pretreatment of the sample prior to analysis. Although the Tina-quant® immunoassay is less precise than the in-house HPLC method, its imprecision is similar to that observed with other immunoassays or automated affinity binding assays (5–13). *Hamwi* et al. (11) and *Lyon* et al. (10) reported slightly lower between-day CV's, between 2.4 and 5.9%, determined however over a shorter period (11 days, n = 11 and 10 days, n = 10 respectively) and using one calibration. The CV's obtained using immunoassays (7, 9–13) often exceed the desired maximum imprecision of 3% for HbA_{1c} (16, 17). The imprecision might be improved by performing duplicate measurements.

The results of the immunoassay were not affected by common interferences like bilirubin, VLDL-rich plasma and labile HbA_{1c}. The labile fraction of glycohaemoglobin reflects the prevalent blood glucose concentration. Failure to efficiently remove this labile fraction prior to the assay causes an apparent increase in % glycohaemoglobin with most methods based on charge separation. Consequently, labile HbA_{1c} does interfere with the HbA_{1c} measurement of HPLC, thus requiring sample pretreatment. The effect of the labile fraction on the Tina-quant® immunoassay was evaluated by in vitro incubation of whole blood specimens supplemented with *D*-glucose (50 mmol/l final). No significant increase of the HbA_{1c} values was observed with the immunoassay which indicates that the assay is insensitive to considerable amounts of labile fraction. Therefore, there is no need to remove the labile fraction prior to the Tina-quant® assay.

The alleged specificity of the antibodies for the glucose moiety of the β-chain N-terminus suggests that the different HbX_{1c}'s investigated should be equally recognized (see package insert). The present study, however, did not allow us to draw any conclusion about the effect of variant Hb's, since some variant containing samples followed the overall correlation with HPLC whereas in others the immunoassay overestimated the apparent HbA_{1c} content compared to HPLC. Further studies are thus required to confirm the antibodies' specificity.

Overall, the results of the immunoassay correlated fairly well with the HPLC method in spite of having a slope of 1.37 with an intercept of −3.72. Haemoglobin A_{1c} values less than 8% were lower with the immunoassay in comparison with HPLC and values above 10% were higher with the immunoassay. *Hamwi* et al. (11) using the Tina-quant® assay and *John* et al. (7) using the Dako enzyme-immunoassay noted a similar bias between immunoassays and HPLC, especially at low HbA_{1c} values. This bias could be a consequence of the higher specificity of the immunoassay for N-terminal glycation as compared to HPLC. Differences in calibration procedures may also contribute to the observed bias (18, 19). In our HPLC assay, HbA_{1c} is determined by expressing the HbA_{1c} peak area as fraction of the total peak area. No calibration was performed. Results obtained by the immunoassay, on the contrary, depend heavily on the calibrator-assigned values. Standardization of the HbA_{1c} measurements by use of common calibrators is essential to improve interlaboratory and between-method consistency (18, 19).

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