

# COMPARATIVE DETERMINATION OF GLYCATED HEMOGLOBIN WITH DIRECT PHOTOMETRIC IMMUNOASSAY AND AFFINITY CHROMATOGRAPHY

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**Summary.** Glycated hemoglobin (HbA1c) is the gold standard in long-term assessment of glycaemic control in patients with diabetes mellitus (DM). Liquid Chromatography-Isotope Dilution-Mass Spectrometry is a reference method for HbA1c, but other methods used in practice need standardization and comparison. This study was aimed to perform a comparative evaluation of the results from HbA1c determination by direct immuno-photometric method (A) and affinity chromatography method (B). The study included 50 samples with values from all clinically measurable areas. HbA1c in whole blood was studied using Olympus AU 400 analyzer and NycoCard READER II by, applying method A and method B, respectively. The results are compared using the program Method Validator, as for the reference method the chromatographic method was accepted. The imprecision in series for method A has been traced in 10 parallel samples from two patients in border and abnormal area of values. The inaccuracy was evaluated in normal and pathological human control material for 25 consecutive days. The results from correlation and regression analysis show linear regression coefficient  $r = 0.949$ , slope = 0.919 and intercept = 0.59. Assessment of imprecision for method A shows CV values of 1.21% and 1.91% for both cases, respectively. Accuracy monitoring of method A with control materials shows d% 4.979 in the normal area, and 5.033 in the pathological area. The results show high positive correlation and comparability between the two methods. Direct immuno-photometric method has good accuracy and precision and is applicable for analyzer Olympus AU 400.

**Key words:** *Glycated haemoglobin, direct immuno-photometric method, affinity chromatography method, NycoCard READER*

## INTRODUCTION

**G**lycated hemoglobin (HbA1c) is considered as a gold standard in long-term assessment of glycaemic control in patients with diabetes mellitus (DM) [11,12,19]. Recently, standardization of hemoglobin HbA1c measurement is of considerable interest, as long as it will provide quality assurance in diabetes management [5,14]. It is recommended by the American Diabetes Association for the diagnosis and screening of type 2 DM. There are over 20 methods of HbA1c determination, based on differences in structure, charge and chemical reactivity, as the first clinically relevant method is described in 1978 [6, 7, 8, 14]. Most commonly used analytical methods are based on high performance liquid chromatography (HPLC), electrophoresis, other chromatographic methods, immune assays, etc. The reference method recommended and accepted in 2007 by the IFCC [9,10] is Liquid Chromatography-Isotope Dilution-Mass Spectrometry (LC-ID-MS HP). Studies establish a difference in the results, which requires standardization and comparison of the methods used in practice. The transition to the recently introduced to practice units (mmol/mol) is part of the standardization and harmonization of the results.

This study was aimed to perform a comparative evaluation of the results from HbA1c determination by direct immuno-photometric method (A) and affinity chromatography (B).

## MATERIALS AND METHODS

The study included 50 patients (25 males and 25 females), aged between 28-55 years. Thirty of them were randomly chosen and 20 were deliberately selected according to values of HbA1c > 8.0%. The HbA1c values ranged from 5.0% to 12.70% and the measurement was performed in accordance with the requirements of the local and international legislation. HbA1c in venous blood was obtained in 3 ml EDTA vacutainers and was kept at 4°C in order to preserve the stability of the samples. The measurement was conducted within 2 days to avoid large differences in the results caused by on-going non-enzymatic glycation, especially at high levels of HbA1c. The studied parameter was measured using Olympus AU 400 analyser, with the reagents, calibrators and controls for method A being produced by PZ CORMAY S.A., Poland. The calibrators for method A are not certified to the IFCC reference method and to the NGSP/DCCT recommended method, but are evaluated using NCCLS protocols [21]. Method A (candidate-method) is based on a direct photometric immunoassay for HbA1c and requires manual preparation of the hemolysate. The hemolysate is obtained by mixing 500 µl hemolysing agent – NaN<sub>3</sub> with 10 µl whole blood stored for 5 minutes or until complete hemolysis occurs in accordance with the testing method procedures. The hemolysate is mixed with latex reagent so that total Hb and HbA1c fraction competitively bound to the latex particles. In the second stage, anti-human HbA1c (mouse) monoclo-

nal antibodies and anti-mouse IgG polyclonal antibodies (goat) are added. Goat anti-mouse antibodies react with mouse anti-human HbA1c–latex complex causing agglutination reaction which is measured photometrically. Linearity for this method, according to the manufacturer, is up to 16%. Method B was performed using Nyco-Card READER II device with reagents and controls produced by Axis-Shield PoC AS, Norway, based on the principle of reflection refractometry. Method B requires 5  $\mu$ L capillary blood or anticoagulated venous blood (EDTA, citrate or heparin) and 3 minutes assay time. It is a fast point of care test based on boronate affinity principle. The calibrator of method B is compared to the primary reference standard, certified September 2011 [22]. The linearity of method B is from 3 to 18%. Both methods interfere with bilirubin up to 50 mg/dl, triglycerides up to 22 mmol/l, HbF up to 30%, haemolysis > 3g/100 ml hemoglobin and ascorbic acid up to 50 mg/dl [16]. Correlation analysis is implemented in the verification of the results and coefficient  $r$  is determined, as a criterion for the strength of the linear relationship between the two groups of comparable variables. Statistical program for regression analysis with Method Validator Software is applied to calculate the Slope, Intercept and  $r$  of the results obtained by both methods [13,18,20]. Because of its standardization, method B (affinity chromatography) was used as a reference method. Verification for significantly deviating values („fugitives”) did not find results > 3.5 SD  $y_x$ , indicating that the random scatter follows a Gaussian distribution. Nevertheless, we focused on Deming regression instead of simple linear regression. Simple linear regression has many limitations and requires the independent variable to be determined without an error, i.e. determining with the reference method. Deming regression takes into account variation of both methods compared.

The imprecision in series for method A has been traced in 10 parallel samples from two patients in the border and the abnormal values. The assessment is conducted using standard deviation (SD) and coefficient of variation (CV).

Evaluation of the inaccuracy of method A is made through monitoring normal and pathological human control material in 25 consecutive days [3]. Inaccuracy is accepted as a measure of total error (systematic + occasional error) and was calculated by formula as  $d\%$ . The absolute deviation (difference) from the target value (Bias) is calculated as the difference between mean and target value of the controls.

Variation analysis and Student t-test were performed using GraphPad Prism software and Interactive Statistical Calculation Pages.

## RESULTS

The values of basic statistical parameters calculated by Method Validator Software are presented in Table 1. The comparison of methods A and B (Fig. 1) showed high positive correlation  $r = 0.949$ . Slope, as a criterion of the proportional error size of the study method A, is 0.919 (0.822 to 1.016). Intercept, a criterion of the size of the constant systematic error of the study method A, is 0.59 (-0.15 to 1.34) and ap-

proaches the ideal value. Figure 2 illustrates HbA1c values measured by methods A and B for each of the patients studied. The application of variation analysis and Student t-test shows no significant statistical differences,  $p > 0.05$ , between the compared methods ( $p = 0.656$ ). Mean  $\pm$  SEM for method A was  $7.85 \pm 0.359$  and for method B was  $7.89 \pm 0.389$  (Fig. 3), and presents the difference plot between the two compared methods. Precision of method A, based on determination of HbA1c in samples of two patients in border and pathological areas, are presented in Table 2 and Table 3. The mean values, standard deviations and coefficient of variance were 6.42%, 0.079, 1.21% and 8.25%, 0.158, 1.91% for the patient in the border area and the patient in the pathological area, respectively. Monitoring of inaccuracy of method A with control material in normal and pathological area in 25 consecutive days demonstrated the following results: for the low control – mean  $\pm$  SEM =  $5.144 \pm 0.066$ ; SD = 0.327; Value for the control material ( $X_0$ ) = 4.9 (3.9-5.9); d% = 4.979; Bias = 0.244. For the high control – mean  $\pm$  SEM =  $11.396 \pm 0.238$ ; SD = 1.167;  $X_0$  = 12.0 (10.9-13.9); d% = 5.033; Bias = -0.604 (Table 4).

**Table 1.** Basic statistical parameters obtained by Method Validator

| n = 50 | “Reference” method B | “Candidate – method” A |
|--------|----------------------|------------------------|
| Mean   | 7.898                | 7.856                  |
| SD     | 2.095                | 1.935                  |
| SEM    | 0.296                | 0.274                  |
| Min    | 5.0                  | 5.0                    |
| Max    | 12.7                 | 12.1                   |

**Table 2.** Results from HbA1c determination in samples of two patients in border and pathological areas

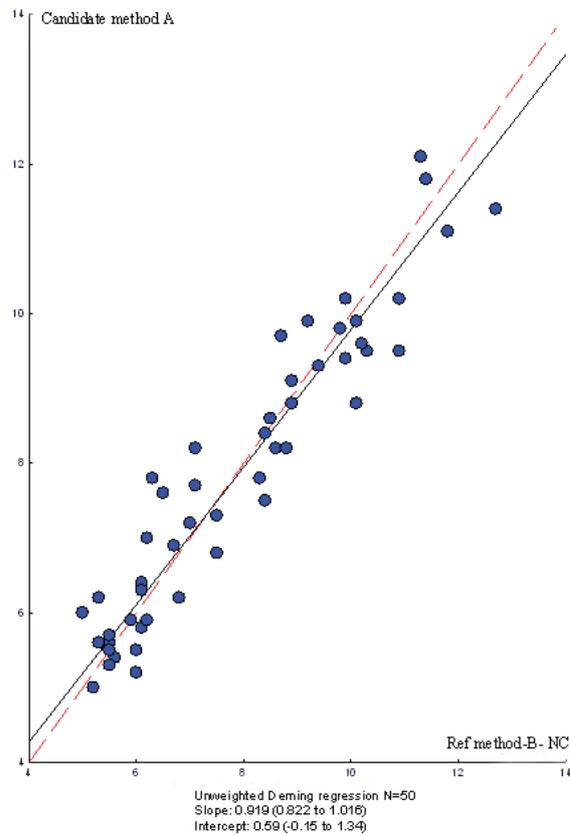
|             | 1   | 2   | 3   | 4   | 5   | 6   | 7   | 8   | 9   | 10  |
|-------------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Patient № 1 | 6.5 | 6.4 | 6.4 | 6.3 | 6.5 | 6.5 | 6.4 | 6.5 | 6.3 | 6.4 |
| Patient № 2 | 8.3 | 8.4 | 8.3 | 7.9 | 8.2 | 8.4 | 8.3 | 8.2 | 8.1 | 8.4 |

**Table 3.** Statistical results for precision in series of method A

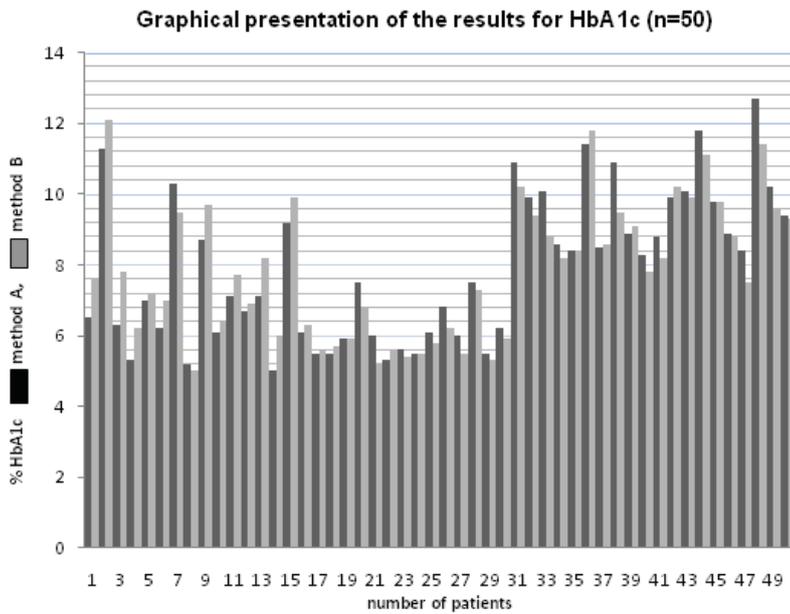
|                   | N  | Mean | SD    | CV   |
|-------------------|----|------|-------|------|
| Border area       | 10 | 6.42 | 0.079 | 1.21 |
| Pathological area | 10 | 8.25 | 0.158 | 1.91 |

**Table 4.** Monitoring of inaccuracy of method A with a control material in normal and pathological area

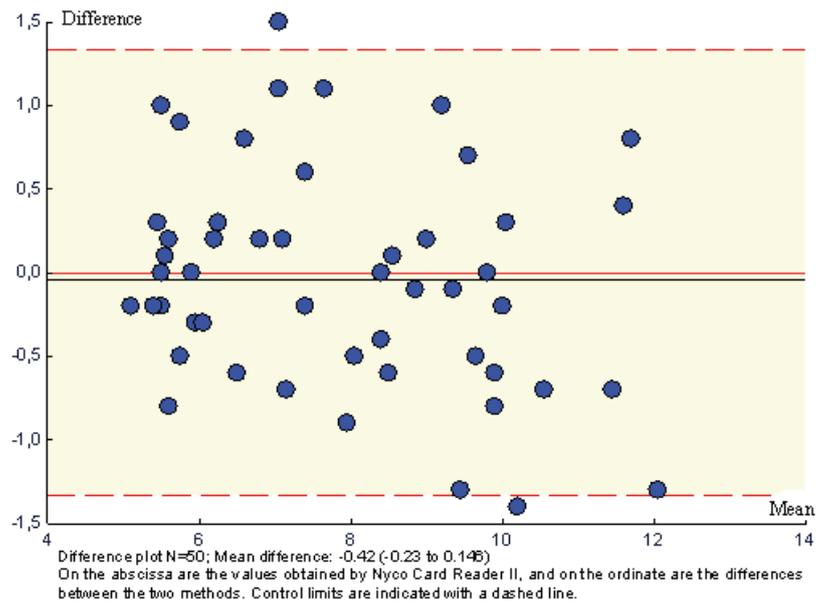
| N = 25          | Mean x | X <sub>0</sub> | Bias   | d%    | SD    | SEM   | CV (%) |
|-----------------|--------|----------------|--------|-------|-------|-------|--------|
| Control level 1 | 5.144  | 4.9            | 0.244  | 4.979 | 0.327 | 0.066 | 6.36   |
| Control Level 2 | 11.396 | 12             | -0.604 | 5.033 | 1.167 | 0.238 | 10.24  |



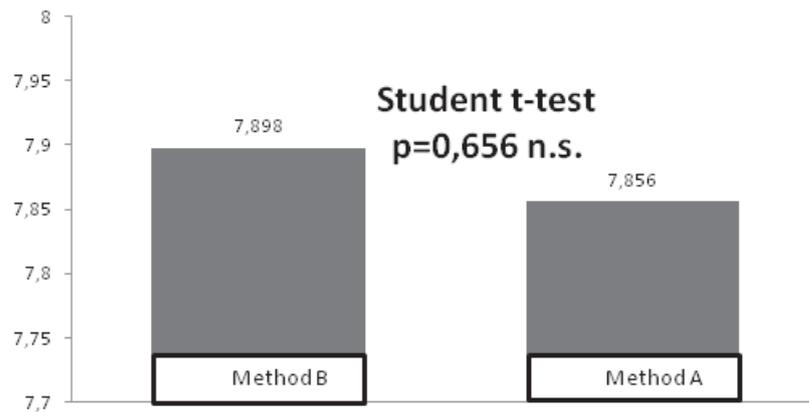
**Fig. 1.** Regression analysis – Deming regression (HbA1c) measured by method A – continuous line and method B – dashed line



**Fig. 2.** Parallel measurement of HbA1c in 50 patients by both methods



**Fig. 3.** Difference plot between the two methods



**Fig. 4.** Comparison of the mean values of HbA1c, assessed by both methods (variation analysis, t-test)

## CONCLUSIONS

Reproducibility (precision) is an assessment of coincidence between the results in identical conditions of repeatability. It is estimated by imprecision, which reflects the dispersion of results around a mean value. Imprecision depends on the random errors size in the analytical stage. The evaluation of imprecision data for HbA1c by method A measured through a direct photometric immunoassay shows  $CV < 2.0\%$ . This result permits the method to be regarded as successfully applicable in clinical practice. The maximal acceptable imprecision for HbA1c is pre-

sented by coefficient of variance less than 6%. According to the Medical Standard of Clinical Laboratory the eligible d% for HbA1c is up to 12% [1, 2]. Method A has d% 4.979 in the normal area and d% 5.033 in the pathological area. The used control materials and the reagent are reliable and traceable in compliance with the storage conditions, dissolving method, proper storage after reconstitution and show good stability of the components. The high correlation coefficient for method A and method B makes them comparable. Comparison between patient results for both methods with Student t-test showed no significant differences ( $p > 0.05$ ). The results from the comparison between the two methods show high correlation and comparability despite the different principles implicated. This perfect relationship is due to standardization of calibrators in accordance with the IFCC reference standard. It should be noted that the preliminary manual sample preparation in pre-analytical phase could be a source of errors in the results.

The direct immune-photometric method has good accuracy and precision. It is applicable for analyzer Olympus AU 400 and it is a good alternative to affinity chromatography.

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