



## Biological variation components of glycated hemoglobin HbA1c on tosoh G7 HPLC

Biological variation components of HbA1c

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### Abstract

Aim: Biological variation (BV) of the HbA1c test can be affected by analysis method, sampling period, and disease activity. The aim of the present study was to examine the impact of CVI, CVA, and II for Tosoh G7 system. Material and Method: 96 samples from 12 healthy volunteers were collected for every two weeks during a two-month period. HbA1c levels of all samples were measured by G7 HPLC (non-porous ion-exchange) Analyser. Biological variation parameters CVI, CVA, CVG, RCV, and II were calculated. Results: The mean of HbA1c was 5.10% (32.3 mmol/mol). CVA was calculated as 2.81%. The CVI and CVG for HbA1c were 1.69% and 7.56%, respectively. RCV and II values were 9.08% and 0.22. Discussion: According to our results, the sampling period and study method were fundamental to determining biological variation parameters. When using the Tosoh G7 system, we observed similar results to other methods, e.g. boronate affinity or immunoturbidimetry. Low individuality index of HbA1c presented that the analyte had marked individuality and that the population-based reference values had low utility for disease monitoring. Thus, estimation and inclusion of RCV in laboratory reports for HbA1c will guide the clinicians in evaluating the importance of difference between serial results and glycemic status of the patients.

### Keywords

HbA1c; Biological Variation Parameters; Tosoh G7 System

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## Introduction

The American Diabetes Association (ADA) has recommended glycated hemoglobin (HbA1c) as a possible alternative for fasting blood glucose in diagnosis of diabetes [1]. According to the suggestions of the ADA, generally HbA1c, fasting plasma glucose, and 2-h plasma glucose during 75-g oral glucose tolerance test are equally appropriate tests for both diabetes screening and diagnosis [2]. After providing the traceability of the existing commercial HbA1c measurement methods to the reference system, analytical targets must be met to ensure the clinical safety of the HbA1c test [3]. The generally accepted method for this purpose is determination of analytical performance target using the biological variation of the analyte of interest. For this reason, it is important to obtain reliable data on biological variation [4].

Biological variation (BV) is defined as an inherent fluctuation that can be described as random variation around the mean concentration during steady-state periods [4]. Biological variation has generally been defined as random fluctuation around the homeostatic set point for each individual (within-subject or intra-individual biological variation) and the differences between the set points of different individuals (between-subject or inter-individual biological variation). These are usually considered as coefficients of variation (CV) and termed CVI and CVG, respectively [5]. Determining the biological variation values and subsequently estimating quality goals of HbA1c are of high importance for optimal patient care [6].

Information about BV is also a prerequisite for the calculation of reference change value (RCV), which is defined as the minimal difference that must be exceeded for a change in two consecutive results in the same individual to become clinically relevant, which depends on both analytical variations (CVA) and within-subject biological variations (CVI) [4]. The clinician will be guided by determining the reference change value (RCV) calculated by considering the analytical systems of HbA1c and variations due to normal biological fluctuations to determine whether the difference between consecutive samples of the individual is indicative of a clinically significant difference.

In the literature there is a wide heterogeneity in data on components of biological variation for HbA1c. In prospective studies, biological variation values of HPLC methods in different devices have been calculated but, to our knowledge, no studies have been performed with the analyser Tosoh G7 (HPLC) to date.

The aim of this study was to estimate the components of biological variation for HbA1c measured by G7 HPLC analyser (Tosoh Bioscience, USA).

## Materials And Method

### Study subjects

The study was initially planned to include 17 healthy participants, but 5 of them were excluded from the study during follow-up due to discontinuing blood sampling. Therefore, the study group consisted of 12 healthy volunteers (9 male, 3 female). The study protocol was approved by the local ethics committee, and written informed consent was obtained from all participants. The inclusion criteria were that the subjects had no predispositions for diabetes, thalassemia syndrome, or other

hemoglobinopathies and, for women, to have regular menstrual cycles and to not be using hormonal contraceptives. Smokers and subjects taking any medication were excluded from the study. Furthermore, fasting plasma glucose and body mass index (BMI) were < 6.0 mmol/L and <30.0 kg/m<sup>2</sup>, respectively, in all subjects.

Venous blood samples were collected on the same day once every two weeks for two months (January to March). Venous blood samples were collected between 09.00 and 09.30 a.m. after 8 h fasting in the sitting position for 1–5 min with minimal stasis by the same skilled phlebotomist to minimize procedural variations. Whole blood specimens were taken into evacuated collection tubes containing K3 EDTA (BD vacutainer) for HbA1c and stored at -80°C until the time of analysis.

### Analytical procedure

HbA1c was measured by G7 HPLC (non-porous ion-exchange) Analyser (Tosoh Bioscience, USA). Analysis was performed without off-line sample pretreatment or interference from Schiff base. The analyser dilutes the whole blood with Hemolysis & Wash Solution, and then injects a small volume of this specimen onto the analytical column. Separation is achieved by utilizing differences in ionic interactions between the cation exchange group on the column resin surface and the hemoglobin components. The hemoglobin fractions are subsequently removed from the column by performing a step-wise elution using the different salt concentrations in the Elution Buffers HSi Variant 1, 2, and 3. The time that passes from injection of the sample to the time the specific peak elutes off the column is called “retention time.” The G7 software has been written so that each of the expected fractions has a window of acceptable retention times. When the designated peak falls within the expected window, the chromatogram peaks will be properly identified. If a peak elutes at a retention time not within a specified window, an unknown peak (POO) is detected. The separated hemoglobin components pass through the LED photometer flow cell where the analyser measures changes in absorbance at 415 nm. The analyser integrates and reduces the raw data, and then calculates the relative percentages of each hemoglobin fraction. The area of the S- Alcis is divided by the sum of the total areas of all peaks up to and including the Ao to obtain a raw S-A1c percentage. This uncorrected result is substituted as the “x” value in the linear regression formula determined during calibration. The analyser prints the final numerical results and plots a chromatogram showing changes in absorbance versus retention time for each peak fraction. The G7 Variant Analysis Mode is NGSP\* certified and the final reportable result is traceable to the Diabetes Control and Complications Trial (DCCT).

All samples were assayed in duplicate, using the analytical system in accordance with the manufacturer’s instructions and checking its alignment before the run by HbA1c control materials according to the manufacturer’s established validation range. The same lots of calibrators, reagent lot- and quality-control materials were used throughout the study and analyses were performed by a single analyst.

### Statistical analysis

Microsoft Office Excel 2007 and ANOVA tests were used for

the statistical analysis. Before performing calculations with the patients' results, the Cochran test was applied to exclude outlying values from the individual subjects, and the Reed test to eliminate mean outlying values. Analytical variation (CVA) was calculated from the duplicate results of each sample. Mean and standard deviation (SD) of the duplicate results were calculated, and the following formula,  $CVA = SD \times 100 / \text{mean}$ , was used to calculate the within-run CVA%.

ANOVA test was used to estimate CVA plus CVI (CVA+I). CVI was calculated by a subtraction step with the two previous variables (CVA+I - CVA).

$$CVI = (CVA + I_2 - CVA_2) / 1/2$$

Between-subject BV (CVG) was obtained by subtracting the CVA+I from the total variation (CVT) found by using all data from all patients.

$$CVG = (CVT_2 - CVI_2 - CVA_2) / 1/2$$

The index of individuality (II) was calculated using the CVI/CVG ratio. The RCVs for the analytes at various probabilities, which were applied to either a single quantity or to two combined quantities, were calculated using the following formula:

$$RCV = 2.17 \times Z \times (CVA_2 + CVI_2) / 1/2$$

Z; probability selected for statistical significance (Z = 1.96 at 95% confidence interval, CI).

### Results

The demographic characteristics and laboratory parameters of healthy subjects are summarized in Table 1. The volunteers' mean of HbA1c was 5.10% (32.3 mmol/mol). The CVI and CVG for HbA1c were 1.69% and 7.56% respectively. CVA was calculated as 2.81%. These results indicated that our data was reliable for calculating BV. The II of the HbA1c was 0.22. When the index is less than 1, two consecutive results from a subject could be outside the RCV but may lie within the population-based reference interval. Thus, the reference values would be of little use, in particular, for deciding whether a significant change had occurred. For this reason, RCV was calculated and found to be 9.08% for HbA1c (at 95% CI). The results of the HbA1c calculations for CVA, CVI, CVG, RCV, and II (CVI/CVG) are presented in Table 2. Additionally, Figure 1 and Figure 2 present the median and absolute (minimum maximum) range of the HbA1c concentrations for each study participant as % and mmol/mol units.

Table 1. The main characteristics of healthy subjects.

Parameter	All group (n=12)	Female (n=3)	Male (n=9)
Age (year) mean±SD	43.5±9.04	34±6.16	45.77±7.87
BMI(kg/m <sup>2</sup> ) mean±SD	23.63±1.3	22.68±1.68	23.95±1.08
HbA1c (%) mean±SD	5.10±0.30	4.82±0.23	5.20±0.25
(mmol/mol) mean±SD	32.33±3.33	29.25 ±2.37	33.36±2.96

### Discussion

Although many studies have been performed with the ion-exchange method, it is important to calculate the device-specific biological variation, because CVA affects all CVI and CVG. The present study is the first one to determine the BV of HbA1c values measured by the Tosoh G7 system. In order to improve analytical targets in HbA1c monitoring, the patterns revealed

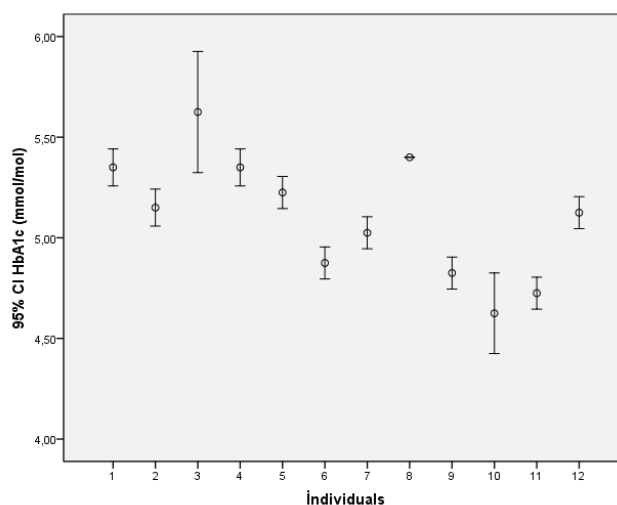


Figure 1. Mean values and absolute range of HbA1c (mmol/mol)

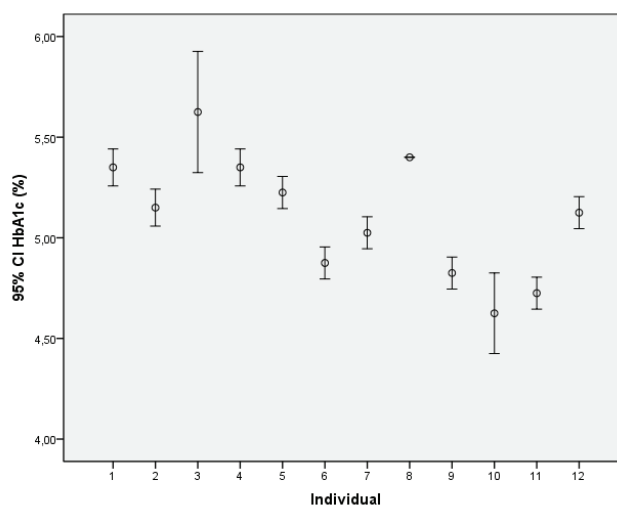


Figure 2. Mean values and absolute range of HbA1c (%)

Table 2. Mean value, estimated average variance components, and indices derived from data on biological variation of HbA1c.

Group	CV <sub>A</sub> %	CV <sub>I</sub> %	CV <sub>G</sub> %	II	RCV%
	2.81	1.69	7.56	0.22	9.08

by Fraser and Harris [7] must be accurately observed and traceability of the HbA1c test with reference material should be ensured. From the analytical point of view, the determination of HbA1c was carried out by the ion-exchange method accepted as the gold standard [8].

Studies conducted with ion-exchange methods revealed similar CV values to our study. The data are presented in Table 3. Kolatkar et al. [6] designed a study in 29 insulin-dependent diabetics for three months sampling interval revealed CVI 2.4%. In another study using ion-exchange method, Carlsen et al. [9] designed a group consisting of 6 female and 9 male between 27 and 59 years of age with a mean time from tip 1 DM diagnosis of 151 months (range 21–408). Researchers evaluated CVI and CVG values of 1.2% and 5.7%, respectively. For a one-year period, Desmeules et al. [10] conducted a study in 38 (24 male, 14 female) pediatric cases with cystic fibrosis but without diabetes or impaired glucose tolerance with the ion-exchange method. In this period they collected five samples from

Table 3. Present and previous studies on biological variability of HbA1c.

	Groups specifics	no. of samples frequency	Mean HbA1c values (%)	Method	CVA (%)	CVI (%)	CVG (%)	RCV (%)	II
Present study	12 (9 M, 3 F, 27–54 years), healthy subjects	2 months, 4 samples	5.10 (32.3 mmol/mol)	HPLC (ion-exchange)	2.81	1.69	7.56	9.08	0.22
Ucar et al. 2013 <sup>(11)</sup>	29 (9 M, 20 F, 20–45 years) healthy subjects	3 months, 4 samples	-	HPLC (ion-exchange)	1.6	1.17	5.58	5.45	0.35
	24 (7 M, 17 F, 20–45 years) healthy subjects			HPLC (boronate-affinity)					
Braga et al. 2011 <sup>(12)</sup>	18 (9 M, 9 F, 26–52 years) healthy subjects	2 months, 5 samples	5.47 (36.3 mmol/mol)	Competitive immunoassay	2.4	2.5	7.1	9.5	0.35
Carlsen et al. 2011 <sup>(9)</sup>	15 (6 M, 9 F) healthy subjects	7 days, 10 samples	5.1	HPLC (ion-exchange)	0.5	1.2	5.7	3.6	-
	15 (9 M, 6 F) type 1 diabetes (2 patients excluded)		7.0		0.6	1.7	8.2	5	-
Desmeules et al. 2010 <sup>(10)</sup>	38 (24 M, 14 F) pediatric with cystic fibrosis but without diabetes or impaired glucose tolerance	1 year, 5 samples	-	HPLC (ion-exchange)	1.4	4.8	12.8	-	-
Rohlfing et al. 2002 <sup>(15)</sup>	45 healthy subjects (only men)	7 days, 12 samples	4.9	HPLC (boronate-affinity)	2.3 (between day)	1.7	4	-	-
Kolatkart et al. 1994 <sup>(6)</sup>	29 insulin-dependent diabetics	3 months, NS	6.5	HPLC (ion-exchange)	1.4	2.4	-	-	-

each patient and calculated CVI and CVG values of 4.8% and 12.8%. For this study, we can see that as the sampling period increases, the CVA increases too, which means that CVI and CVG are affected. The CVI calculated for HbA1c was 4.8% and the between-subject variation (CVG) was 12.8%. In the present study we collected samples from healthy volunteers at intervals of two weeks over the course of two months. As in the previous studies we calculated CVI as 1.69%. Our CVG values were higher than in the other studies (7.56%). Studies that are conducted with the boronate affinity method had different results than those designed with ion-exchange. Ucar et al. [11] calculated CVI and CVG as 2.15% and 4.03%. During 7 days and for 12 samples in 45 healthy men CVI and CVG values were reported as 1.7% and 4%. With the boronate affinity method CVG values are lower than with ion-exchange chromatography. A design similar to our study was conducted by Braga et al. [12] which used the competitive immunoassay method. They followed 18 healthy volunteers for two months and collected five samples from each subject and calculated CVI as 2.5% and CVG as 7.1%. When we analyzed these robust data, it is clear that the BV of the HbA1c test was affected by sampling interval and periods.

The index of individuality (II) parameter gives information about the appropriateness of the evaluation of test results according to reference ranges. A value of II <1 points out high individuality which means that the test results of an individual should be interpreted according to his/her own previous test results instead of pre-set reference values. The individuality indices were found to be 0.35 and 0.93 for ion-exchange chromatography and boronate affinity chromatography, respectively, by Ucar et al.[11] Other studies of individuality indices for ion-exchange chromatography found II values as 0.27 and 0.96 by Kilpatric et al.[13] and Garde et al.[14], respectively. Similarly, the calculated II

values for HbA1c in our study were 0.22 (Table 3). This value demonstrated that evaluation of HbA1c test results should not be made according to population-based reference values.

RCV, also an important parameter in BV for many analytes, could be calculated using BV data from a healthy population. RCV is defined as the critical difference that must be exceeded between two sequential analyses from the same patient. RCV is used when the results from consecutive measurements from the same patient fall within the reference range but represent a considerable change, which indicates a change in the patient's condition [4]. Ucar et al.[11] estimated RCV (critical difference) as 5.45% for ion-exchange chromatography and 10.40% for boronate affinity chromatography; another study conducted by Braga et al. [12] found RCV (critical difference) as 9.5%. These studies investigated how RCV and results are affected by different methods. RCV values measured by HPLC were found to be about 5% lower than by boronate affinity. For the immunoturbidimetric method, these values vary by up to 30% (Table 3). These results indicate that when calculating RCV we should carefully notice the method that was used.

All of the estimations [minimum, desirable and optimal analytical goals for imprecision, bias, total error] were calculated based on data about CVI, CVG, and CVA for the ion-exchange method (Table 4).

Table 4. Quality specifications for HbA1c measurement derived from our data on biological variation

Quality level	Imprecision, %	Bias,%	TE <sub>a</sub> %
Optimal	<0.42	<0.95	<1.64
Desirable	<0.63	<1.91	<2.94
Minimal	<1.26	<2.87	<3.90

In conclusion, knowledge about BV parameters and RCV values for HbA1c might be important for the optimum use of this test in clinical laboratories and reliable calculation of analytical performance. This preliminary research contributes to the literature on BV data and calculation of RCV for HbA1c measured by the G7 HPLC analyser and may be improved with a follow-up study including a larger, gender-matched participant group.

#### Scientific Responsibility Statement

*The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.*

#### Animal and human rights statement

*All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.*

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#### Conflict of interest

*None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.*

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