Original papers

The influence of sample freezing at – 80 °C for 2–12 weeks on glycated haemoglobin (HbA$_{1c}$) concentration assayed by HPLC method on Bio-Rad D-10® auto analyzer

Katarzyna Bergmann*, Grazyna Sypniewska

Department of Laboratory Medicine, Nicolaus Copernicus University Collegium Medicum, Bydgoszcz, Poland

*Corresponding author: bergmann@vp.pl

Abstract

Introduction: The aim of the study was to evaluate the effect of a single freeze/thaw cycle on HbA$_{1c}$ concentrations measured by commercially available HPLC method.

Materials and methods: Study included 128 whole blood samples collected from diabetic patients (N = 60) and healthy volunteers (N = 68). HbA$_{1c}$ concentrations were measured in fresh blood samples. Then samples were frozen at - 80 °C for up to 12 weeks. HbA$_{1c}$ was assayed by ion-exchange HPLC method on Bio-Rad D-10® analyzer. Variables were compared using Wilcoxon and ANOVA Kruskal-Wallis tests. Bias between HbA$_{1c}$ measured in fresh and frozen samples was calculated. The comparability of HbA$_{1c}$ concentrations was assessed by Bland-Altman plot.

Results: Median (IQR) HbA$_{1c}$ concentration was 45.3 (36.6−61.2) mmol/mol for fresh and 45.3 (36.6−60.6) mmol/mol for frozen/thawed samples. No significant difference in HbA$_{1c}$ concentrations was found comparing fresh and frozen/thawed samples (P = 0.070) in the whole group, as well as in healthy and diabetic subjects. The median calculated bias between fresh and frozen/thawed samples was 0% in whole group and healthy subjects, and 1.19% in diabetic patients. No significant difference was found between the biases according to baseline HbA1c values (P = 0.150). The Bland-Altman plot analysis showed a positive bias of 0.4% (95% CI: - 2.8 - 3.7%), which indicates high compliance between HbA$_{1c}$ values and no relevant influence of sample freezing on clinical significance of HbA$_{1c}$ measurement.

Conclusions: Storage for up to 12 weeks at – 80 °C with a single freeze/thaw cycle does not affect HbA$_{1c}$ concentrations measured with HPLC method on Bio-Rad D-10® analyzer.

Key words: glycated haemoglobin; diabetes; sample storage; high performance liquid chromatography; preanalytical phase

Introduction

Glycated haemoglobin (HbA$_{1c}$) is a product of the non-enzymatic binding of glucose to N-terminal valine residues of haemoglobin's β chains. It is considered a "gold standard" for monitoring diabetes, however current guidelines of the American Diabetes Association (ADA) recommend its use as a diagnostic tool for diabetes (1). HbA$_{1c}$ concentration is strongly correlated with the development of long-term complications (micro- and macroangiopathy) in both Type 1 and Type 2 diabetes (2, 3).

Changes in the structure and chemical-physical properties of haemoglobin have become the starting point for the development of various methods for its quantification. Many in vitro diagnostic manufacturers developed automatic analyzers based on enzymatic, immunological and separation methods for HbA$_{1c}$ assay. According to the standardization programme of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC), determination of HbA$_{1c}$ concentration should be performed using high performance liquid chromatography (HPLC) combined with capillary electrophoresis or mass-spectrometry (4). Autoanalyzers and methods used in clinical practice are obliged to obtain IFCC certification.

Glycated haemoglobin concentration is stable in blood during short-term storage, which is its main advantage over glucose (5). Usually, it is measured in fresh samples immediately after blood collection or in samples stored at 4 °C up to 1–2 weeks. Bio-Rad D-10® Hemoglobin A1c Program (Bio-Rad Laboratories, CA, USA) is based on ion-exchange HPLC method which is certified by IFCC. According to the manufacturer, whole blood samples are stable up to 7 days when stored at 2–8 °C or 3 days at room temperature (15–30 °C). Sample freezing is not recommended (6).

Several studies evaluated repeatability of HbA$_{1c}$ concentration on different instruments after freezing the blood samples which might be useful if prolonged storage is required (i.e. for research or evaluation of new methods). For prolonged storage (several months or years) recommended storage temperatures are -70 °C or lower (7-9). HbA$_{1c}$ concentration is stable at 4 °C usually for 2–3 weeks, while at room temperature relevant changes in HbA$_{1c}$ concentration are observed within 1–3 days (8, 9).

The aim of this study was to evaluate the effect of a 2–12 weeks storage period and single freeze/thaw cycle on HbA$_{1c}$ concentrations measured by HPLC on Bio-Rad D-10® autoanalyzer in clinically healthy and diabetic individuals. Most studies evaluating the influence of sample storage on HbA$_{1c}$ concentration were performed using very small numbers of analysed samples. For example, in studies mentioned above only 3 and 5 whole blood samples were analyzed, which can considerably limit their clinical importance (8, 9). Moreover, many studies do not distinguish results obtained in healthy and diabetic subjects. In this study, we made an attempt to compare indicators of the analytical quality and recommended bias related to clinical significance of HbA$_{1c}$ measurements.

Materials and methods

Subjects

This applied research included 128 whole blood samples collected from 60 Caucasian diabetic patients (median 62 (29–81) years) and 68 Caucasian healthy volunteers (median 41 (18–72) years). Subjects were selected based on questionnaire concerning medical history and medical examination performed in the Department of Laboratory Medicine at Nicolaus Copernicus University Collegium Medicum in Bydgoszcz, Poland. The diabetic group consisted of patients with diagnosed Type 1 or Type 2 diabetes. The control group consisted of apparently healthy, non-obese subjects with normal fasting plasma glucose (3.3–5.5 mmol/L) and without chronic diseases in medical history (comprising diabetes, impaired fasting glucose or impaired glucose tolerance, dyslipidemia, autoimmune and chronic infectious diseases, cardiovascular disease – previously diagnosed hypertension, atherosclerosis, coronary heart disease, myocardial infarction, stroke, peripheral artery disease, venous thrombosis).

Written informed consent from each participant was obtained and the study was approved by the Bioethics Committee at Nicolaus Copernicus University Collegium Medicum in Bydgoszcz, Poland.

Blood sampling

From each subject, one sample of fasting venous blood was drawn in the morning (7.00–9.00 am) using sterile 2.0 mL plastic tubes Vacutainer® with potassium - EDTA (cat. no. 367841; Becton Dickinson, NJ, USA). Samples were delivered to the laboratory and HbA$_{1c}$ concentrations were measured immediately after collection in fresh blood samples. Several samples (approximately 16–18) that could not be tested immediately after collection (for example, blood was drawn on Sunday) were stored at 2–8 °C for no longer than 24 hours. Samples were then transferred into sterile, tightly closed polypropylen tubes (cat. no. 20-1203-1; Medlab-Products, Raszyn, Poland) suitable for freezing up to –90 °C to avoid peptide degradation and potential interference with the material of blood collection tubes, and frozen at –80 °C. Samples were stored for up to 12 weeks.

Samples were divided according to the three storage time intervals: 2–5 weeks (N = 40), 6–9 weeks (N = 45) and 10-12 weeks (N = 43). This division was

used to obtain comparable size groups and similar number of diabetic and healthy subjects matched by age, sex and baseline HbA$_{1c}$ concentration in each storage time interval.

Samples were thawed in the refrigerator, brought to room temperature and thoroughly mixed on a roller mixer (MX-T6-S, Medlab-Products, Raszyn, Poland) prior to the assay.

Methods

HbA$_{1c}$ was assayed by ion-exchange high performance liquid chromatography (HPLC) method certified by National Glycohemoglobin Standardization Program (NGSP) and IFCC on Bio-Rad D-10® (Bio-Rad Laboratories, CA, USA) autoanalyzer with reportable range 18 - 179 mmol/mol (3.8–18.5%) and total precision CV = 1.16%. The instrument automatically dilutes whole blood samples and injects them into the analytical cartridge. In the next step, different fractions of haemoglobin are separated using buffer gradient of increasing ionic strength. Each type of haemoglobin is measured by spectrophotometry at 415 nm.

Results were obtained in NGSP units (%). To convert results to IFCC units (mmol/mol), the following equation was used: IFCC = (10.93 x NGSP) - 23.50 (4).

The quality control of this assay was performed every day during the study using Bio-Rad Lyphochek® Diabetes Control with the following reference values: level 1 (low) 5.4%–42.1 mmol/mol) and level 2 (high) 9.6%; ranged 8.4–10.8% (81.4 mmol/mol; ranged 68.3–94.5 mmol/mol). The intra-assay variation calculated from 10 assays of the same sample (in duplicates) was 0.87% and the inter-assay variation calculated from 10 runs on duplicate samples was 1.47%. The intra- and inter-assay variations were performed using quality control material at level 1.

Statistical analysis

Statistical analysis was performed using Statistica 12.0 PL (StatSoft Inc. 2014, Oklahoma, USA) and MedCalc 16.2.1 (MedCalc Software 2016, Ostend, Belgium) for Windows. The normality of distribu-tion was evaluated by Shapiro-Wilks test. All variables had non-Gaussian distribution and were compared using Wilcoxon matched pairs test (comparison between two groups) and ANOVA Kruskal-Wallis test (comparison of more than two groups). The concordance of HbA$_{1c}$ values was assessed by Bland-Altman plot. P < 0.05 was considered significant.

To evaluate the significance of changes in HbA$_{1c}$ concentrations after sample freezing, the repeatability was calculated. Repeatability was considered as absolute difference between HbA$_{1c}$ concentration measured in fresh blood samples and in frozen/thawed samples. A difference of 5 mmol/mol (0.5%) in HbA$_{1c}$ concentration between samples was considered clinically significant.

Bias between HbA$_{1c}$ in fresh and frozen samples was calculated using the following formula: Bias (%) = [(HbA$_{1c}$ fresh - HbA$_{1c}$ frozen) / HbA$_{1c}$ fresh] x 100%. According to the recent findings from "Guideline-Driven Medical Decision Limits" bias less than 1.0% is recommended to obtain clinically useful HbA$_{1c}$ results (10).

Results

Median of HbA$_{1c}$ concentration was 45.3 (36.6 - 61.2) mmol/mol (6.3 (5.5–7.8)%) for all fresh samples and 45.3 (36.6–60.6) mmol/mol (6.3 (5.5–7.7)%) for frozen/thawed samples (P = 0.070). In healthy subjects median of HbA$_{1c}$ concentration was 37.2 (34.4–43.1) mmol/mol for fresh and 36.9 (34.4–43.1) mmol/mol for frozen/thawed samples (P = 0.062). Similarly, no significant differences were observed in patients with diabetes (P = 0.086).

Repeatability of HbA$_{1c}$ concentration after single freeze/thaw cycle is presented in Figure 1. No changes in HbA$_{1c}$ concentration was found in 48.4% of subjects (N = 62), while in 41.4% cases (N = 53) the obtained results were lower (mean 1.4 mmol/mol) and in 10.1% cases (N = 13) the results were higher (mean 2.3 mmol/mol) than baseline HbA$_{1c}$ values, respectively. However, the observed differences in HbA$_{1c}$ concentrations between fresh and frozen/thawed samples were not statistically significant (P = 0.110).
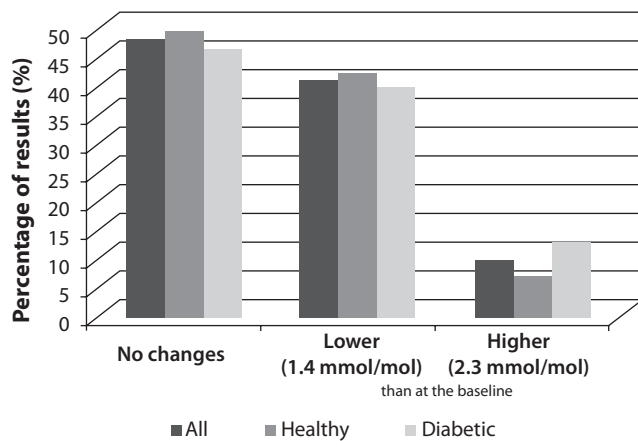
Figure 1. Repeatability of Hb$_{A1c}$ concentrations after single freeze/thaw cycle. A difference of 5 mmol/mol (0.5%) in HbA$_{1c}$ concentration between samples was considered as significant change.

Samples were divided according to storage time: 2–5, 6–9 and 10–12 weeks, respectively (Table 1). No significant differences in HbA$_{1c}$ concentration were found comparing frozen/thawed samples stored in different time intervals when compared to fresh blood samples.

Samples were divided according to baseline HbA$_{1c}$ values into three groups: < 39 mmol/mol (< 5.7%), 39 - 47 mmol/mol (5.7–6.4%) and ≥ 48 mmol/mol (≥ 6.5%). No significant differences were observed between HbA$_{1c}$ concentration in fresh and frozen/thawed samples in each group (Table 2).

The Bland-Altman plot analysis (Figure 2) showed a positive bias of 0.4% (95% CI: -2.8–3.7%), which indicates high compliance between HbA$_{1c}$ values measured in fresh and frozen/thawed samples.

Table 1. Effect of storage time on HbA$_{1c}$ concentration in frozen/thawed samples

Storage time, weeks	HbA$_{1c}$ concentration, mmol/mol		
	Fresh samples	Frozen/thawed samples	P
2–5 weeks (N = 40)	36.1 (34.4–58.5)	36.0 (34.4–58.5)	0.270
6–9 weeks (N = 45)	45.3 (38.8–61.7)	46.4 (37.7–48.4)	0.390
10–12 weeks (N = 43)	45.3 (36.6–55.2)	45.3 (36.1–54.1)	0.130

Results are presented as median and interquartile range (IQR). Difference in HbA$_{1c}$ concentrations between fresh and frozen/thawed samples were teste using the Wilcoxon test. P<0.05 was considered statistically significant.

Table 2. HbA$_{1c}$ concentration in fresh and frozen/thawed samples divided according to baseline HbA$_{1c}$ concentrations

Baseline HbA$_{1c}$ concentration, mmol/mol	HbA$_{1c}$ concentration, mmol/mol		
	Fresh samples	Frozen/thawed samples	P
< 39 (N = 32)	34.4 (33.3–36.6)	34.4 (32.2–36.6)	0.150
39 - 47 (N = 38)	45.3 (42.1–46.4)	44.2 (42.1–46.4)	0.650
≥ 48 (N = 58)	66.1 (55.2–79.2)	67.2 (55.2–79.2)	0.250

Results are presented as median and interquartile range (IQR). Difference in HbA$_{1c}$ concentrations between fresh and frozen/thawed samples were teste using the Wilcoxon test. P<0.05 was considered statistically significant.
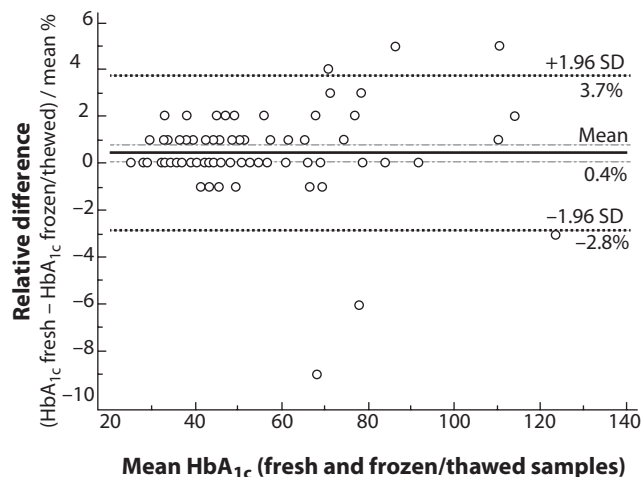
Figure 2. Bland-Altman plot of differences in HbA$_{1c}$ concentrations in fresh and frozen/thawed samples. Solid line (Mean) – mean difference. Grey lines – 95% confidence intervals of mean difference. Dashed lines (SD) – standard deviation.

The median calculated bias between fresh and frozen samples was 0% in whole group and in healthy subjects (interquartile range (IQR): 0–1.64%) and 1.19% (IQR: 0–2.15%) in diabetic patients, respectively. No significant difference was found between the biases according to the baseline HbA$_{1c}$ values (P = 0.150; Figure 3).
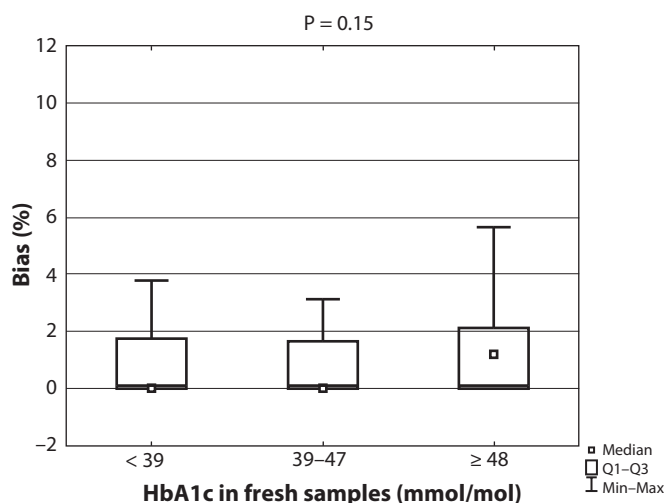

Figure 3. Comparison of bias values according to baseline HbA1c concentration (ANOVA Kruskal-Wallis test). Q1 – first quartile. Q3 – third quartile. P < 0.05 was considered statistically significant.

Discussion

We demonstrated good stability of HbA$_{1c}$ measured in whole blood samples stored for 2–12 weeks at −80 °C. Comparison of HbA$_{1c}$ concentration in fresh and frozen/thawed samples indicates high compliance, independently of storage time and baseline HbA$_{1c}$ concentrations.

A study performed in France showed that HbA$_{1c}$ concentration assayed by ion-exchange HPLC method (Bio-Rad Variant II®; Bio-Rad Laboratories, CA, USA) is stable at −80 °C for at least one year and for 2 weeks when stored at 4 °C (9). However, substantial HbA$_{1c}$ degradation occurred at −20 °C after 15 days of storage; therefore, this temperature is not recommended for HbA$_{1c}$ sample storage. Similar results were observed in a study by Little *et al*. They found that HbA$_{1c}$ measured on five different HPLC autoanalyzers was the most stable at −70 °C (for a storage period of 57 days), while in four methods stability at 4 °C was higher than at −20 °C (14–57 *vs*. 3 - 28 days, respectively) (11). Although HbA$_{1c}$ stability was analysed in different temperature and storage time periods, the relevance of the results appears to be significantly limited due to very low number of samples (only three samples in each study).

Storage at very low temperature (−70 °C or lower) might be useful also in case of quality control materials. Marron *et al*. compared imprecision of Lyphocheck® Diabetes Control Bilevel (Bio-Rad Laboratories, CA, USA) measured on HPLC instrument (12). Control material was stored up to 7 days in 2–8 °C and alternatively frozen in 10 µL aliquots at −80 °C. The coefficient of variation (CV) obtained by standard procedure were 2.33% and 2.51% for level 1 and 2, respectively; while the CV obtained by alternative procedure were significantly lower: 1.93% (P < 0.001) and 0.99% (P = 0.003). Moreover, control materials freezing reduced costs of quality control by 80%. Optimization of quality control and cost saving may be important especially in small-scale laboratories, where HbA$_{1c}$ measurements are not performed every day.

Although freezing seems to be an effective way to store blood samples for HPLC methods, some au-

thors consider that long-term storage may increase HbA$_{1c}$ concentration assayed by mass spectrometry methods. D'Alessandro *et al.* observed relative percentage increase in HbA$_{1c}$ concentration in samples frozen for 28–42 days compared with samples stored up to 14 days, however the authors did not clearly state the freezing temperature (13). This effect was explained by use of additive solutions, such as SAGM (saline-adenine-glucose-mannitol) for blood banking which results in prolonged exposure of erythrocytes to high glucose concentrations. However, their study group consisted of only 10 healthy volunteers, thus the results should be interpreted with caution. The influence of SAGM on HbA1c concentrations should be verified in samples obtained from patients with diabetes or elderly subjects, who have increased rate of glycation.

Recent studies suggest that repeatability of HbA$_{1c}$ measurements depends on baseline HbA$_{1c}$ concentration in fresh blood samples. In a study by Liotta *et al.*, including 237 subjects with type 2 diabetes, HbA$_{1c}$ levels measured by ion-exchange HPLC method were compared in fresh samples and samples stored for 1 year at −80 °C (14). HbA$_{1c}$ concentrations were significantly decreased after freezing and refreezing in patients with lower (< 6.5%), increased (6.5–8.0%) and very high (> 8.0%) baseline concentrations. Moreover, bias was significantly higher in subjects with HbA$_{1c}$ ≥ 6.5% compared with those with HbA$_{1c}$ < 6.5%, and increasing bias values were observed in subsequent concentration intervals of baseline HbA$_{1c}$. However, our results did not confirm this tendency in both diabetic and healthy subjects.

In the Atherosclerosis Risk in Communities (ARIC) study HbA$_{1c}$ concentration in 14 069 whole blood samples was measured by HPLC method after storage for 14 and 18 years at −70 °C. The correlation between measurements at the two time points was high; however a slight positive bias of 0.29% was found when comparing samples stored for 18 years to those stored for 14 years (15).

For HbA$_{1c}$ measurement used in particular as a diagnostic tool for diabetes diagnosis, evaluation of diabetes risk in general population or establishing therapeutic goals in diabetic patients the corresponding values of analytical quality indicators must be maintained. According to generally accepted rule in clinical practice, difference of 5 mmol/mol (0.5%) in HbA$_{1c}$ concentration between patients samples is considered as a significant change in glycemic control (16, 17). When comparing this criterion with the repeatability observed in our study, it can be concluded that single freeze/thaw cycle does not affect the clinical significance of HbA$_{1c}$ measurement. However, the evaluation of clinical significance of HbA$_{1c}$ concentrations should also consider the analytical bias of assay used. A recent study by Hyltoft Petersen and Klee, which appraises the influence of analytical bias and imprecision on the number of false positive results, recommends bias less than 1.0% to obtain clinically useful HbA$_{1c}$ results (10). In our study a slight positive bias of 0.4% between HbA$_{1c}$ values in fresh and frozen/thawed samples was found in Bland-Altman plot analysis. Thus, high compliance of results obtained in both diabetic and healthy individuals was demonstrated, with no significant effect on HbA$_{1c}$ measured from samples stored at -80 °C. Several studies indicate different bias of HbA$_{1c}$ concentration assayed by comparable methods. For example, study performed in Denmark proposed a more restrictive bias (i.e. less than 0.3%), which provides acceptable accuracy for the detection of significant changes in glycemic control and risk prediction of diabetic complications (18).

Despite potentially interesting findings, we should emphasize the important limitations of this study. First, study consisted of relatively small number of subjects and storage time was relatively short, therefore results require confirmation in extended population-based study. Moreover, the time between blood collection, HbA$_{1c}$ assay and samples freezing should be unified. Almost all samples were delivered to the laboratory, measured and frozen immediately after collection; however, several of them were stored at 4 °C and assayed not later than within 24 hours, which may possibly induce some variation in HbA$_{1c}$ concentration. However, it is worth emphasizing that our study included significantly greater number of analyzed sam-

ples, when compared to previously published research. Moreover, it is one of the few studies which evaluated the stability of HbA$_{1c}$ concentration during freezing in both healthy and diabetic subjects. Obtained results indicate that sample storage at – 80 °C does not affect the clinical significance of HbA$_{1c}$ measurement on HPLC autoanalyzer, therefore this method of storage might be crucial for maintaining analytical quality of assay, as well as for its cost-effectiveness.

In conclusion, whole blood samples storage at - 80 °C up to 12 weeks and a single freeze/thaw cycle do not affect the concentration of HbA$_{1c}$ measured by HPLC method on Bio-Rad D-10® analyzer in healthy and diabetic subjects. However, due to relatively short storage period studied, results should be confirmed by measuring HbA$_{1c}$ levels in long-term stored samples.

Potential conflict of interest

None declared.

References

1. *American Diabetes Association. Standards in medical care in diabetes - 2015. Diabetes Care 2015;38:S1-93.*

2. *The Diabetes Control and Complications Trial Research Group. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. N Engl J Med 1993;329:977–86. http://dx.doi.org/10.1056/NEJM199309303291401.*

3. *UK Prospective Diabetes Study (UKPDS) Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes (UKPDS 33). Lancet 1998;352:837–53. http://dx.doi.org/10.1016/S0140-6736(98)07019-6.*

4. *Hoelzel W, Weykamp C, Jeppsson JO, Miedema K, Barr JR, Goodall I, et al. IFCC reference system for measurement of hemoglobin A1c in human blood and the national standardization schemes in the United States, Japan, and Sweden: a method-comparison study. Clin Chem 2004;50:166–74. http://dx.doi.org/10.1373/clinchem.2003.024802.*

5. *Rohlfing C, Wiedmeyer HM, Little R, Grotz VL, Tennill A, England J, et al. Biological variation of glycohemoglobin. Clin Chem 2002;48:1116-8.*

6. *Bio-Rad D-10® Hemoglobin A1c Program Instruction Manual. Available at http://www.bio-rad.com. Accessed December 20th 2015.*

7. *Selvin E, Coresh J, Jordahl J, Boland L, Steffes MW. Stability of haemoglobin A1c (HbA1c) measurements from frozen whole blood samples stored for over a decade. Diabet Med 2005;22:1726-30. http://dx.doi.org/10.1111/j.1464-5491.2005.01705.x.*

8. *Rohlfing CL, Hanson S, Tennill AL, Little RR. Effects of whole blood storage on hemoglobin A1c measurements with five current assay methods. Diabetes Technol Ther 2012;14:271-5. http://dx.doi.org/10.1089/dia.2011.0136.*

9. *Szymezak J, Lavalard E, Leroy N, Gillery P. Incidence of sample storage temperature on HbA1c determination by high performance liquid chromatography method. Ann Biol Clin (Paris) 2009;67:705-10.*

10. *Hyltoft Petersen P, Klee GG. Influence of analytical bias and imprecision on the number of false positive results using Guideline-Driven Medical Decision Limits. Clin Chim Acta 2014;430:1-8. http://dx.doi.org/10.1016/j.cca.2013.12.014.*

11. *Little RR, Rohlfing CL, Tennill AL, Connolly S, Hanson S. Effects of sample storage conditions on glycated hemoglobin measurement: evaluation of five different high performance liquid chromatography methods. Diabetes Technol Ther 2007;9:36-42. http://dx.doi.org/10.1089/dia.2006.0055.*

12. *Marrón PI, Soria PR, Ramos PA, Moreno MA. Optimization of imprecision and cost saving on hemoglobin A 1c controls through aliquot freezing. Clin Chem Lab Med 2008;46:1199. http://dx.doi.org/10.1515/CCLM.2008.236.*

13. *D'Alessandro A, Mirasole C, Zolla L. Haemoglobin glycation (Hb1Ac) increases during red blood cell storage: a MALDI-TOF mass-spectrometry-based investigation. Vox Sang 2013;105:177-80. http://dx.doi.org/10.1111/vox.12029.*

14. *Liotta L, Di Franco A, Pazzagli M, Luconi M. Glycated haemoglobin (HbA1c) measurement in frozen whole blood depends on baseline values of fresh samples. Anal Bioanal Chem 2013;405:429-34. http://dx.doi.org/10.1007/s00216-012-6480-y.*

15. *Selvin E, Coresh J, Zhu H, Folsom A, Steffes MW. Measurement of HbA1c from stored whole blood samples in the Atherosclerosis Risk in Communities study. J Diabetes 2010;2:118-24. http://dx.doi.org/10.1111/j.1753-0407.2010.00070.x.*

16. *Weykamp C. HbA1c: a review of analytical and clinical aspects. Ann Lab Med 2013;33:393-400. http://dx.doi.org/10.3343/alm.2013.33.6.393.*

17. *Little RR, Rohlfing CL, Sacks DB. Status of hemoglobin A1c measurement and goals for improvement: from chaos to order for improving diabetes care. Clin Chem 2011;57:205-14. http://dx.doi.org/10.1373/clinchem.2010.148841.*

18. *Jørgensen LG, Brandslund I, Stahl M, Hyltoft Petersen P, Iversen S, Klitgaard N et al. Upper reference limit, analytical quality specifications and clinical use of haemoglobin A1C. Scand J Clin Lab Invest 2002;62:609-22. http://dx.doi.org/10.1080/003655102764654349.*