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TECHNICAL NOTE

Glycated hemoglobin (HbA1c) measurement in frozen whole blood depends on baseline values of fresh samples

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Abstract Glycated hemoglobin (HbA1c) has been recently adopted as a diagnostic marker of type 2 diabetes. However, its usage is currently limited to fresh blood samples. To allow retrospective HbA1c measurement in blood banks developed in large epidemic studies, here, we contribute to validate HbA1c assessment in frozen versus fresh blood samples from a cohort of diabetic/nondiabetic adult subjects. HbA1c was measured by HPLC in 237 fresh whole blood samples and on the same samples after a 12-month storage and a further 6-month-refrozen storage. Mean HbA1c±SD in fresh, frozen, and refrozen samples was 6.9±1.2, 6.6±1.1, and 6.4±1.0 % for the Diabetes Control and Complications Trial and 52±13, 49±12, and 46±11 mmol/mol for the International Federation of Clinical Chemistry and Laboratory Medicine reference, respectively. A significant correlation was found between fresh/frozen and fresh/refrozen (R=0.994 and 0.993, P< 0.001) samples. HbA1c relative error ratio (%RER) between frozen/refrozen and fresh samples significantly correlated with HbA1c and depended on fresh value range, increasing in the five HbA1c classes (<6.0, 6.0–6.5, 6.5–7, 7–8, ≥8 %, corresponding to <42, 42–48, 48–53, 53–64, ≥64 mmol/mol, P<0.001). In particular, the 6.5 % (48 mmol/mol) HbA1c diagnostic cutoff of fresh samples identified two classes reflecting significant differences in %RER (2.8±2.0 and 3.3±1.7; P<0.05) between frozen and fresh samples. In conclusion, our results demonstrate a high correlation between data from fresh and frozen samples, with a very limited %RER between the two measurements, which increases with baseline HbA1c levels. Accordingly, when analyzing biobank frozen specimens for diagnostic purpose, the effect of the HbA1c range should be taken into account.

Keywords Glycated hemoglobin · Diabetes · Biobank · Whole blood · Frozen

Introduction

After its introduction in the early 1980s into clinical usage as a marker of long-term glycemic control in people affected by type 2 diabetes (T2D), glycated hemoglobin (HbA1c) has been recently adopted by the World Health Organization [1] and by the International Expert Committee guidelines [2] as a laboratory tool for the diagnosis of the world's most prevalent metabolic disease [3]. However, much debate is ongoing on the appropriateness of this marker for the diagnosis of overt diabetes (HbA1c≥6.5 % for the Diabetes Control and Complications Trial (DCCT) and ≥48 mmol/mol for the International Federation of Clinical Chemistry and Laboratory Medicine, IFCC) [2–5]. The HbA1c value expresses the percentage of HbA1c on adult normal hemoglobin (HbA) for the DCCT definition or the ratio between HbA1c/HbA [millimoles per mole] according to the new IFCC reference [6] and strictly correlates with the average blood glucose levels during the previous 3 months [7].

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Compared with other markers used for T2D diagnosis and provided that measurements are calibrated to international standards and no condition impeding accurate measurement are present [5, 8, 9], HbA1c usage displays several advantages. Among these, the most important can be considered a great pre-analytical stability of the measure, no need of fasting condition, a less intra-individual variability, and the necessity of a single sample. A variety of methods has been proposed since the beginning for evaluation of HbA1c in blood ranging from fresh to frozen and dried samples [10–14]. A validated international reference method for HbA1c measurement in human blood has been developed by IFCC, defining the standard reference values for the analytical characteristics of HbA1c routine assay [15].

Despite very few studies assessing HbA1c analysis on frozen samples [10–13], a comprehensive study evaluating reliability of HbA1c measurements in frozen compared to the same fresh whole blood samples from a large cohort of nondiabetic and diabetic adults is still needed.

Materials and methods

Patients

EDTA–whole blood samples were drawn from 237 patients attending the Diabetes and Obesity Agency at our university hospital (AOUC), who gave their written informed consent. The study sample included men and women with undiagnosed/diagnosed T2D at first visit or during follow-up under treatment for the disease. This enables us to assess the validity of the method across a wide range of HbA1c values.

Samples and study design

HbA1c was freshly evaluated in 2 h from blood collection. The remaining blood was frozen at −80 °C controlled temperature. After 1 year of storage, samples were thawed, HbA1c was evaluated on the same instrument (*frozen* samples), and the remaining whole blood was refrozen and kept for an additional 6 months at −80 °C controlled temperature. Finally, samples were rethawed (*refrozen* samples) and analyzed on the same HPLC instrument.

HbA1c assay

Glycated hemoglobin (HbA1c) measurement was performed on whole blood samples by a HPLC ion exchange chromatography on an ADAMS A1C HA8160 instrument (Menarini Diagnostic, Florence, Italy). The instrument was validated for routine analysis [16, 17] for diagnostic purpose on the IFCC reference method [15]. The HA8160 instrument is a fully automated HPLC analyzer working on anticoagulant-added whole blood samples to measure HbA1c (range, 3–20 % for the DCCT system; 9–195 mmol/mol for the IFCC system). Three microliters of the automatically diluted (1:100 for standard and 1:10 for

Table 1 HbA1c values in fresh, frozen, and refrozen groups and their relative correlations in samples stratified according to fresh HbA1c values

HbA1c classes in fresh samples	Age (years)	Fresh (%) (mmol/mol)	Frozen (%) (mmol/mol)	*R*	*P*	Refrozen (%) (mmol/mol)	*R*	*P*
TOT (*n*=237)	67.7±12.1	6.9±1.2 52±13	6.6±1.1 49±12	0.994	<0.001	6.4±1.0 46±11	0.993	<0.001
<6.5 % (<48 mmol/mol) (*n*=104)	67.6±11.7	6.0±0.4 42±4	5.8±0.3 40±4	0.931	<0.001	5.7±0.3 39±4	0.934	<0.001
≥6.5 % (≥48 mmol/mol) (*n*=133)	66.7±12.4	7.6±1.2 60±13	7.3±1.1 56±12	0.993	<0.001	7.0±1.0 53±11	0.992	<0.001
<6.0 % (<42 mmol/mol) (*n*=43)	66.0±13.0	5.7±0.3 39±3	5.5±0.3 37±3	0.924	<0.001	5.4±0.3 36±3	0.915	<0.001
6.0–6.5 % (42–48 mmol/mol) (*n*=61)	68.7±10.7	6.2±0.1 44±1	6.0±0.2 42±2	0.706	<0.001	5.9±0.2 41±2	0.729	<0.001
6.5–7.0 % (48–53 mmol/mol) (*n*=55)	67.7±11.3	6.7±0.1 50±2	6.5±0.2 48±2	0.636	<0.001	6.2±0.2 44±2	0.687	<0.001
7.0–8.0 % (53–64 mmol/mol) (*n*=44)	68.8±11.3	7.4±0.3 57±3	7.2±0.3 55±3	0.912	<0.001	6.9±0.3 52±3	0.895	<0.001
≥8.0 % (≥64 mmol/mol) (*n*=34)	62.2±14.6	9.2±1.2 77±13	8.8±1.1 73±12	0.994	<0.001	8.4±1.1 68±12	0.992	<0.001

Mean±SD are indicated as well as the number of samples per class (*n*). HbA1c values are expressed according to the DCCT (in percent) or IFCC (in millimoles per mole) reference. Student's *t* test analysis for paired samples indicated a statistically significant difference ($P<0.001$, not shown) between fresh/frozen and fresh/refrozen HbA1c values in all classes. Multivariate regression parameters (*R*, *P*) are indicated for correlation between fresh and frozen/refrozen samples after age adjustment

anemic samples, respectively) sample was injected on an analytical prefilter-equipped column, packed with an ion exchange resin (a hydrophilic polymer of methacrylate ester copolymer). Elution is achieved in a five-step phosphate-buffered gradient with increasing ionic strength. HbA1c fractions are detected with a dual wavelength of 420–500 nm LED photodiode. At this wavelength, the absorption of oxy- and deoxyhemoglobin is equivalent with a stable signal independent of ratio between the two forms.

Within-run and between-run CV are 1.5 and 1.5 % at low HbA1c concentrations and 0.9 and 1.0 % at high HbA1c concentrations, respectively [16, 17], with imprecision less than the 2 % level standardized by the IFCC reference method [15]. Trueness has a maximal deviation from the target of 1 %mmol/mol (IFCC system) and 0.1 % (DCCT system) throughout the analytical working range, as evaluated in the federate program national EQA [17]. Linearity, carryover, and linear drift are excellent and in the range required by the IFCC reference method [15]. Labile-HbA1c, carbamylated hemoglobin, variations in bilirubin and hematocrit, and the presence of hemoglobin variants AS, AC, and F do not affect HbA1c measurement.

The routine HbA1c analysis undergoes a regular certified national external quality control program (VEQ, Italian Society of Clinical Biochemistry, SIBioC).

Statistical methods

Data are expressed as mean±SD. One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was applied for multiple comparisons, whereas two-tailed Student's *t* test was used for two classes of data. Pearson's correlation was used when appropriate. Stepwise multiple linear regressions were applied for multivariate analysis. All statistical analyses were performed using the SPSS 18.0 software for Windows.

Results and discussion

Hb1Ac evaluation was performed in all fresh and frozen samples with the automated HPLC instrument HA-8160 (Menarini Diagnostic, Italy) and expressed versus HbA content as percent or as millimoles per mole according to the DCCP or IFCC systems. We calculated the reproducibility of our instrument during the period in which we

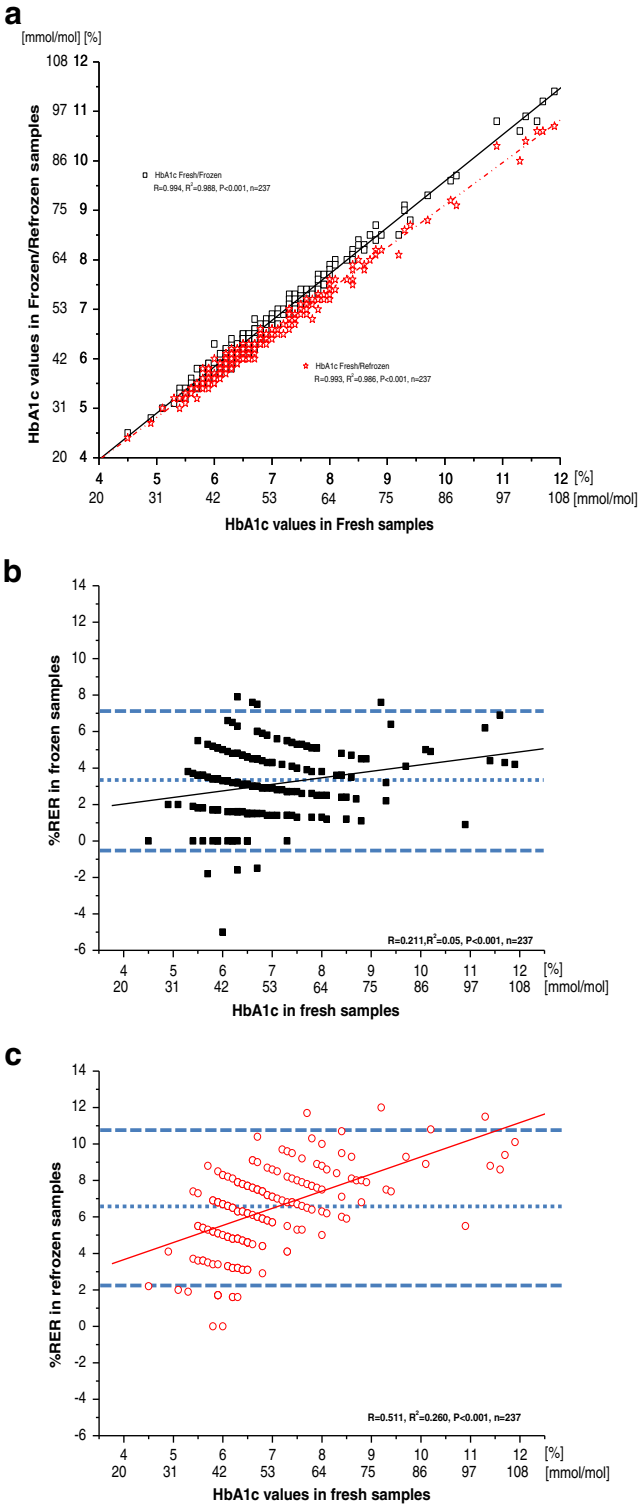

Fig. 1 Correlation between fresh values and HbA1c measurements or %RER in frozen and refrozen blood samples. **a** HbA1c data were stratified according to fresh HbA1c values for frozen (*open squares*) or refrozen (*open stars*) samples. **b**, **c** %RER mean values as well as CI (mean±2 SD) are indicated as *dotted* and *dashed lines*, respectively, in frozen (**b**) and refrozen (**c**) samples. The *solid line* indicates linear regression curve. *R*, R^2, *n*, and *P* values obtained by multivariate analysis after age adjustment are indicated. HbA1c values are expressed according to DCCT (in percent) or IFCC (millimoles per mole) reference

Table 2 Relative error ratio of HbA1c measurements in frozen (%RER frozen) and refrozen (%RER refrozen) samples stratified according to fresh values and their correlations with HbA1c values in fresh samples

Fresh HbA1c classes	%RER frozen	*R*	*P*	%RER refrozen	*R*	*P*
TOT (*n*=237)	3.09±1.92	0.211	<0.001	6.39±2.19	0.511	<0.001
<6.5 % (<48 mmol/mol) (*n*=104)	2.82±2.03	0.156	ns	5.25±2.03	0.181	ns
≥6.5 % (≥48 mmol/mol) (*n*=133)	3.31±1.81	0.230	0.009	7.29±1.88	0.406	<0.001
<6.0 % (<42 mmol/mol) (*n*=43)	2.54±1.76	0.219	ns	4.93±2.03	0.262	ns
6.0–6.5 % (42–48 mmol/mol) (*n*=61)	3.01±2.20	0.036	ns	5.46±2.03	0.061	ns
6.5–7.0 % (48–53 mmol/mol) (*n*=55)	3.07±2.05	0.204	ns	6.59±1.90	0.119	ns
7.0–8.0 % (53–64 mmol/mol) (*n*=44)	3.37±1.53	0.160	ns	7.38±1.57	0.232	ns
≥8.0 % (≥64 mmol/mol) (*n*=34)	3.60±1.73	0.424	0.010	8.29±1.78	0.307	ns

Mean±SD is indicated as well as the number of samples per class (*n*). Student's *t* test analysis for paired samples showed a statistically significant difference between %RER in frozen and refrozen samples in all classes ($P<0.001$, not shown). Multivariate regression analysis parameters (*R*, *P*) are indicated for the correlation between %RER frozen/refrozen and HbA1c in fresh samples, after age adjustment

ns not significant

performed the study by assessing HbA1c values on at least ten samples assayed in duplicate within the same run in the same day (intra-assay) and between runs in two consecutive days (inter-assay). The obtained CVs were: intra-assay, 0.06 and 0.08 % for low and high HbA1c concentrations (5.8 and 7.5 % or 40 and 58 mmol/mol), and inter-assay, 1.8 and 2.0 % for low and high HbA1c concentrations (5.0 and 9.1 % or 31 and 76 mmol/mol). These results were in accordance with the IFCC standard reference method [15].

Hb1Ac was normally distributed in fresh, frozen, and refrozen samples (Electronic supplementary material, Fig. S1) independently of gender and significantly decreased after the first (frozen) and the second (refrozen sample) freezing (Table 1). When samples were stratified according to fresh HbA1c values in two (diagnostic cutoff of 6.5 %–48 mmol/mol) or in five classes (HbA1c<6.0, 6.0–6.5, 6.5–7.0, 7.0–8.0, and ≥8.0 % corresponding to <42, 42–48, 48–53, 53–64, and ≥64 mmol/mol), differences in HbA1c measurements between fresh, frozen, and refrozen samples were statistically significant between all classes (Table 1).

After age adjustment, a significant positive correlation existed between frozen and fresh HbA1c as well as between refrozen and fresh HbA1c (Fig. 1a). The slope of the regression curve slightly decreased between frozen ($B=0.938$) and refrozen samples ($B=0.858$), suggesting a systematical decrease in HbA1c measurement following each freezing step (Table 1). Correlations between fresh and frozen HbA1c remained significant even when fresh samples were split in the two or five classes of fresh HbA1c (Table 1), supporting the relative stability of HbA1c values after midterm storage

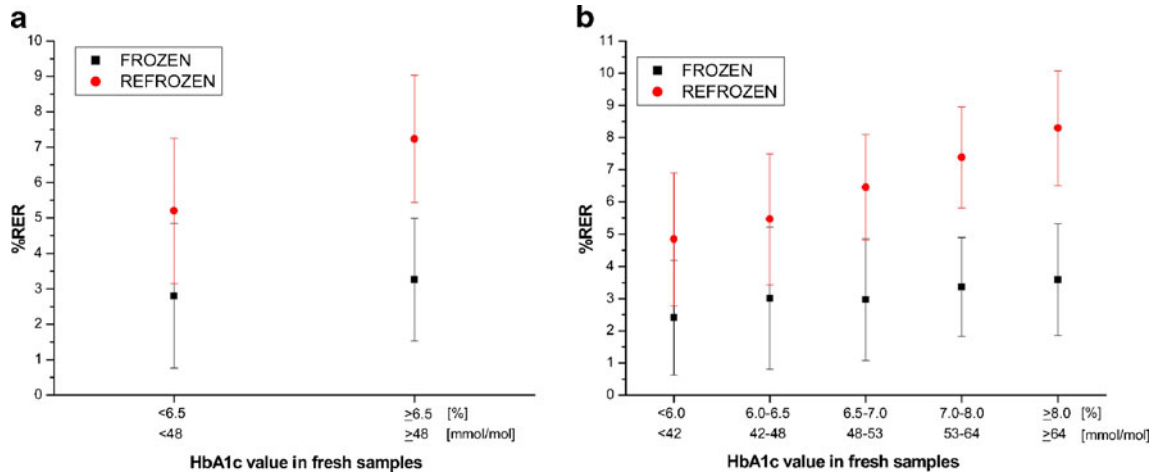

Fig. 2 Distribution of %RER mean in the fresh HbA1c classes. %RER mean±SD increases significantly for increasing fresh HbA1c values in both frozen and refrozen samples. HbA1c values are expressed according to DCCT (in percent) or IFCC (millimoles per mole) reference. **a** Student's *t* test for unpaired samples has been applied for comparison between two classes of fresh HbA1c (cutoff=6.5 %–48 mmol/mol), $P<0.05$ and $P<0.001$, for frozen and refrozen samples, respectively. **b** ANOVA followed by Bonferroni's post hoc test has been applied for comparison between the five classes of fresh HbA1c: $F=1.8$, $P<0.05$ and $F=21.9$, $P<0.001$, for frozen and refrozen samples, respectively

as well as on refrozen samples in a wide range of fresh HbA1c. Correlation coefficients of regression curves are very close between fresh and frozen/refrozen samples (0.994 and 0.993, respectively) and quite comparable with those obtained by other studies [10–13].

Reduction in HbA1c measurements after freezing can be expressed either as absolute delta value [ADV=Δ(fresh−frozen)] or relative error ratio percent on frozen versus fresh measurements [%RER=100×Δ(fresh−frozen)/fresh]. Although ADV is the most common difference measurement reported in the literature [10, 11, 13] and indicates the reduction of the absolute measurement of HbA1c after freezing, it does not take into account the baseline HbA1c level. Conversely, %RER considers the difference in the measurement in relation to fresh HbA1c value.

A multivariate regression analysis after age adjustment (Table 2) revealed a significant positive correlation between %RER of frozen (Fig. 1b)/refrozen (Fig. 1c) samples and fresh HbA1c values. The slope of the regression curves increased from frozen (B=0.386) to refrozen (B=0.940) samples, indicating that error increases at each freezing step (Fig. 1b, c).

When stratified in classes, such correlation retains a statistical significance for pathological classes only (≥6.5 %–48 mmol/mol and ≥8 %–64 mmol/mol), suggesting that the error on HbA1c measurement significantly affects high HbA1c values only (Table 2). %RER increased significantly for increasing fresh HbA1c values in both frozen (Fig. 2a) and refrozen (Fig. 2b) samples in all classes of stratification. These findings indicate that higher values of HbA1c tend to result in a statistically significant greater error when measurements are performed after one or multiple freezing, suggesting a higher rate of degradation in richer samples. This is the first time that the influence of HbA1c range of fresh samples on HbA1c measurement in frozen ones is considered. Which are the molecular bases underlying this phenomenon is still unknown. HbA1c is generated inside the red cells by the nonenzymatic glycation process of the N-terminal valine on the HbA beta chain in a two-step Maillard reaction. First, glucose forms a labile and reversible aldamine (Schiff base) with the N-terminal valine on the beta chain. This step is followed by an Amadori rearrangement of aldamine to form a stable ketoamine. It could be hypothesized that the freezing/thawing process may affect the Kd of the first reversible step in an HbA1c concentration-dependent manner, resulting in greater shift of the equilibrium towards resolution of the aldamine intermediate in HbA1c richer samples. Alternatively, HbA1c underestimation in the richer frozen samples may occur due to a direct effect of the freezing/thawing procedure on the second step. The influence of hemolysis in affecting HbA1c measurement in frozen samples has been previously excluded [13]. However, specific alteration in total as well as in HbA levels following freezing/thawing process, which can affect the relative HbA1c value in frozen samples, cannot be excluded. Interestingly, a recent study retrospectively demonstrated the stability of long-term stored blood samples for the new method of glycated albumin evaluation, through validation of this stability for glycated hemoglobin measurements in fresh compared to long-term-stored samples [18]. Further studies, conducted even on different blood products of glycation, such as albumin, are needed to elucidate this critical point.

Conclusion

In conclusion, our data reinforce the reliability of HbA1c evaluation in midterm frozen blood samples, pointing out, however, the possible influence of baseline value range in affecting the percentage of error in stored samples. All these findings strongly contribute to support the validity of retrospective analyses of glycated hemoglobin in historical series of frozen blood from various epidemiological studies, enabling us to improve the number of possible predictive parameters to be retrospectively studied.

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