Assessment of Hemoglobin A2 stability at room temperature during 24 or 25 days as measured by high pressure liquid chromatography and capillary electrophoresis

Dear Editors,
As a de facto reference laboratory, we receive samples from most parts of the country for hemoglobinopathy evaluation. These samples may be days or even weeks old before they reach our laboratory, due to mail transport as well as handling and analysis in other laboratories. We cannot control the conditions for transport and storage before the samples arrive in our laboratory, and it is important for our evaluation to know how these factors may affect the results of the relevant analyses. We have previously investigated how long-time storage affects hematology parameters, which are also integral to diagnosing heterozygous β-thalassemia. In this paper, we investigated long-term stability of hemoglobin fractions analyzed by both HPLC and CE in samples stored at room temperature for up to 24 or 25 days after initial testing.

The hemoglobin (Hb) A molecule consists of two α- and two β-globin subunits and is the primary Hb molecule after one year of age. In addition, Hb contains a minor fraction, HbA2, consisting of two α- and two δ-globin subunits. In the erythropoiesis of people heterozygous for β-thalassemia, the production of normal HbA is decreased, and there is a relative increase in production of HbA2 in some cases HbF. Heterozygous β-thalassemia causes mild, microcytic anemia and obtaining a diagnosis is relevant both with regard to treatment, prevention and counselling. The diagnosis is supported by an increased fraction of HbA2 in hemolyzed blood.

The trial was accepted by the hospital Data Protection office, reference number 19/02589. Surplus material from routine testing of venous samples with di-potassium ethylenediaminetetraacetic acid as anticoagulant was used. Samples were collected during September – November 2019 in the Department of Medical Biochemistry, Oslo University Hospital based on hematology results from XN 9000 (Sysmex, Kobe, Japan), partly values that might be compatible with thalassemia, that is low MCH and MCV and partly samples with normal MCH and MCV. This yielded 20 samples with elevated HbA2 (>3.4%) and 30 samples with normal HbA2 (<3.4%). Due to a missing data point, one out of the 30 samples was excluded, leaving 29 samples with normal HbA2 for analysis. The samples were anonymized before inclusion in the study. Hemoglobin fractionation was performed first time between 0 and 3 days after sample collection (except for one sample at 4 days after collection), and samples were stored at room temperature thereafter. All samples were then tested repeatedly at 3 to 4 days intervals, to a total of 8 tests per sample during a 24- or 25-day period. All samples were on each occasion analyzed by high performance liquid chromatography (HPLC) using BioRad Variant 2 (Hercules, CA) with the Beta-Thal short program, and by Capillary electrophoresis (CE) on Capillaries 3 Tera (Sebia, Lissex, France) by the Capillaries Hemoglobin assay.

Data for all fractions from both methods were plotted and analyzed in MS Excel 2016 and IBM statistical package for the social sciences (SPSS) statistics 26. Results were analyzed in four groups: normal HbA2, analyzed by CE (normal HbA2/CE); normal HbA2, analyzed by HPLC (normal HbA2/HPLC); increased HbA2, analyzed by CE (high HbA2/CE) and; increased HbA2, analyzed by HPLC (high HbA2/HPLC). All HbA2 concentrations were normalized according to results of daily internal controls. HbA2 results for each of the four groups were tested for normality using Shapiro-Wilk test and Kolmogorov-Smirnov test which both demonstrated data not to be normally distributed. Friedman test was therefore utilized to assess change in the HbA2 concentrations during the period. A Wilcoxon post hoc analysis was also used to compare sequential time points. Chosen significance level for all tests was P < .05, but due to multiple comparisons a Bonferroni-adjustment was used, yielding an adjusted p-value of 0.0016. Wilcoxon test was also used for comparing relative HbA2 levels between HPLC and CE. For this, we chose P < .05 as significant for each comparison, since we looked for sustained differences during sequential days and not individual time points.

We obtained higher values for HbA2 fractions by HPLC than by CE, with mean bias of 0.60% and 0.32% of total hemoglobin for the high HbA2 and normal HbA2 groups, respectively.

The HbA2 fractions decreased with time in samples from both the high HbA2 and the normal HbA2 groups and this reduction in HbA2 was observed after analysis by both methods (P < .001, Friedman test, for all four groups, Figure 1A-D). By Wilcoxon post hoc test, the change in HbA2 from day 0 was statistically significant already at day 3/4, except in the high HbA2/CE group, in which...
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HbA2 reduction was statistically significant at day 7. Thereafter, HbA2-concentration tended to stabilize from day 7 to day 24/25, at a median level of approximately 90% of start value, except for the high HbA2/HPLC group where median HbA2 stabilized at a level of approximately 80% of start value. We observed a considerable variation between samples in the relative reduction of HbA2 with storage time (Figure 1).

HbA2-concentrations of the two initially distinct groups of high and normal HbA2 converged slightly as time passed, and the separation between the groups became somewhat blurred (Figure 2). When comparing HPLC and CE in the corresponding HbA2 groups using Wilcoxon-test, we found significantly lower HbA2 reduction with time for CE than for HPLC at day 14 and later time points for the high HbA2 group (P < .05 for all time points), while there was no sustained statistical difference between methods for the normal HbA2 group.

With increasing time of storage, several small, new peaks appeared for both CE and HPLC, probably reflecting degradation (Figure 3).

The most important finding in our study was that the HbA2 concentrations as analyzed by both CE and HPLC were reduced during the first 3 to 7 days of storage, but then tended to stabilize at a median level of 80%-90% of initial value. For all four groups, the relative reduction in HbA2 concentration showed considerable variation between different samples, especially at later time points, rendering interpretation of results more difficult. Bento et al.5 investigated HbA2 stability for seven days in capillary blood stored at room temperature, using HPLC (BioRad Variant II), measuring at day 3, 5, and 7. They concluded that HbA2 was stable for seven days. Mercadanti et al.6 investigated HbA2 stability during storage at 4°C and -80°C for a week and found HbA2 to be stable under these conditions. HbA1c-equipment was utilized in 13 patients with a range of hemoglobinopathies, at temperatures ranging from 4 to 50°C up to 15 days. This study found most hemoglobins to be stable at 15 days at 37 degrees, but significantly reduced stability at higher temperatures. To the best of our knowledge, no previous study has compared HbA2 stability between CE and HPLC. Furthermore, HbA2 stability at room temperature for as long as 24/25 days has not been investigated before.

We found that the HbA2 values were reduced with storage at room temperature. Samples with HbA2 concentrations that were initially above the "diagnostic gray zone" of approximately 3.2 to 3.6%, in some cases after storage showed HbA2 concentrations within this range, as shown in Figure 2.

This demonstrated that older samples should be interpreted with caution and that the time to analysis should be taken into consideration. Nevertheless, as no sample at any time point showed an increase above start value in measured HbA2 fraction larger than 0.1% of total Hb, our data indicate that a high HbA2 measurement is reliable also in old samples. A normal HbA2 result derived from an old sample, however, should be interpreted cautiously if used to exclude heterozygous β-thalassemia.

**FIGURE 1** Percentage of initial value for 20 samples with high HbA2 (A and C) and 29 samples with normal HbA2 (B and D) from day 0 to day 24/25 analyzed by CE (A and B) and by HPLC (C and D). Box and whiskers plots illustrate median, quartiles and range as well as outliers at each time point. Outliers are defined as dots when outside of 1.5 interquartile range, and crosses when outside of 3 interquartile ranges.
FIGURE 2  Dot-plot of HbA2 values as percent of total hemoglobin measured at each time point as indicated by CE (A) and HPLC (B). Red dots denote increased HbA2 at day 0, blue dots denote normal HbA2 at day 0.
The observed significantly lower HbA2 reduction for CE than for HPLC at the later time points for the high HbA2 group, suggests that CE could be preferable to HPLC in old samples. However, since the bias between instruments was larger in the high HbA2 group than in the normal HbA2 group, these two effects would counteract each other.

This study is partially limited by the fact that the first analysis was performed at different times from collection between samples. This was due to practical limitations at the laboratory and a consequence of using surplus blood from routine testing. Therefore, day 0 referred to different dates for different samples, and the time from collection to first analysis varied between 0 and 3 days (4 in one sample).

Due to sample volume limitations from using surplus material, we chose to study stability at room temperature only. This clearly does not perfectly reflect transport conditions, but from preliminary studies we knew that HbA2 was more stable at lower temperatures (O. Klingenberg, unpublished data) and higher temperatures are not often encountered in Norway.

This study is the first to compare HbA2 values between HPLC and CE in samples stored up to 25 days at room temperature. This is relevant for assessment of blood samples delayed in transport from patients with suspected heterozygous β-thalassemia. We found that median HbA2 is significantly reduced after 3-7 days, tending to stabilize at a level of 80%-90% after day 7. Importantly, we found that the HbA2 fractions of some samples were reduced with time to such an extent that the diagnosis of heterozygous β-thalassemia could be missed. This, and multiple degradation spikes found at later time points, underlines the need for vigilance in the laboratory when investigating old samples. However, our study also demonstrates that HbA2 does not increase with storage, so high HbA2-concentrations may be trusted.
KEYWORDS
beta-thalassemia, capillary electrophoresis, hemoglobinopathies, high pressure liquid chromatography, thalassemia

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The authors have no competing interests.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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REFERENCES
1. Sudmann-Day AA, Piehler A, Klingenberg O, Urdal P. Six-day stability of erythrocyte and reticulocyte parameters in-vitro: a comparison of blood samples from healthy, iron-deficient, and thalassemic individuals. Scand J Clin Lab Invest. 2015;75:247-253.
2. Weatherall DJ. Phenotype-genotype relationships in monogenic disease: lessons from the thalassaemias. Nat Rev Genet. 2001;2:245-255.
3. Higgs DR, Engel JD, Stamatoyannopoulos G. Thalassaemia. Lancet. 2012;379:373-383.
4. Old JM. Screening and genetic diagnosis of haemoglobin disorders. Blood Rev. 2003;17:43-53.
5. Bento C, Relvas L, Vazao H, Campos J, Rebelo U, Ribeiro ML. The use of capillary blood samples in a large scale screening approach for the detection of beta-thalassemia and hemoglobin variants. Haematologica. 2006;91:1565.
6. Mercadanti M, Romero A, Lippi G. The measurement of hemoglobin A2 and F in stored whole blood samples. Clin Lab. 2011;57:777-780.
7. Colah RB, Wadia M, D’Souza E, Sawant PM, Mohanty D, Mehra M. Evaluation of a capillary blood collection system for screening for hemoglobinopathies in remote areas. Int J Lab Hematol. 2010;32:e57-63.

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