Measurements of Free Hemoglobin and Hemolysis Index: EDTA- or Lithium-Heparinate Plasma?

There is no agreement as to the optimal sample type to be used for measurements of free hemoglobin (fHb) in plasma. (Because fHb is increased during clotting, serum is not recommended.) Several publications have recommended the use of EDTA as an anticoagulant (2), but another author reported 20-fold higher fHb values in EDTA-plasma than in Li-heparinate plasma and therefore recommended Li-heparinate plasma for such analyses (1). Because Li-heparinate and EDTA plasma are often used for emergency and routine measurements, we explored this issue and searched for a practicable, inexpensive, and rapid method for fHb measurements.

Blood from 49 patients was collected into sample tubes (Monovette, Sarstedt) containing EDTA (1.6 g/L of whole blood) or Li-heparinate (16 kIE/L of whole blood). Samples were collected from patients who required laboratory analyses. Patients gave informed consent, and the study received institutional review board approval. The P-module of the Modular5 (Roche), which was preinstalled to determine serum/plasma indices, was used to measure the hemolysis index (HI). For comparison with the HI, we used the 2-wavelength method of Golf et al. (4) to measure fHb and not the more commonly used method of Harboe (5), because measurements at multiple wavelengths are not available for users on the Modular. Briefly, fHb was determined by using the difference of absorbances at 540 and 600nm. A hemoglobin solution, prepared as described by Fairbanks et al. (2), was used as a calibrator at 1250 mg/L. The method detection limit was approximately 50 mg/L as determined by analysis of replicate measurements (n = 10) of calibrator dilutions and the 0 calibrator. At concentrations of 90, 300, 1250 and 9500 mg/L, intraassay imprecision values (CVs) for fHb were 9%, 5.6%, 1.3%, and 0.5%, respectively, and for HI were 15%, 3.6%, 0.6%, and 0.3%. Interassay CVs, determined by replicate measurements during 10 days at 90 and 1250 mg/L fHb concentrations, were 9.4% and 3.5% for fHb and 12.4% and 0.6% for H-index measurements, respectively. The values for fHb in our patients ranged from <50 to 794 mg/L in EDTA plasma and from <50 to 1120 mg/L in Li-heparinate plasma. The corresponding values for H-index ranged from <50 to 760 (EDTA plasma) and <50 to 1070 mg/L (Li-heparinate plasma), respectively. In univariate ANOVA, no significant differences in mean (SD) fHb values were observed between specimens collected in EDTA [232 (179) mg/L] or heparin [254 (226) mg/L]. Similarly, mean (SD) HI did not differ between the 2 materials [153 (173) for EDTA and 173 (225) for Li-heparinate]. Correlation coefficients, R, between fHb and H-index were 0.939 in EDTA plasma and 0.967 in Li-heparinate plasma. Difference plots (fHb – HI) for both plasma collections showed no correlation of difference with the mean of fHb and HI. Correlations between fHb and HI in EDTA plasma were substantiated in 200 additional samples (R = 0.990). In these samples, values for fHb and HI ranged from <50 to 6300 mg/L and <50 to 7030 mg/L, respectively. In samples with fHb values <900 mg/L, the correlation remained high (0.860). We therefore could not confirm with these sample collection tubes the statement that fHb may be 20-fold higher in EDTA plasma than in heparin plasma (1).

An effect of EDTA became apparent when we determined fHb and HI in blood from healthy volunteers collected with increasing EDTA concentrations. At EDTA concentrations 3 times the usual, both fHb and HI were >50% higher than values in EDTA plasma collected under standard conditions. In contrast, similar experiments with Li-heparinate plasma showed no dependence of fHb or HI on Li-heparinate concentrations in the range of 16–48 kIE/L whole blood. Thus EDTA influences fHb and HI in a concentration-dependent manner, but the effect can...
be disregarded when collection tubes are correctly filled.

We conclude that fHb and HI can be measured in blood collected either with heparin or EDTA provided that EDTA tubes are adequately filled. Because of the excellent agreement of values for fHb measurements and HI in the P-module of the Modular, HI may be sufficient in many clinical settings.

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References

Hemoglobin Hagley Park: A Novel (α82Ala→Thr) Substitution Identified in an Infant with Severe Hemolytic Anemia

To the Editor:
The identification of new hemoglobin mutations and the correlation of structural changes with potential pathology continue to provide insights into how normal structure and function are preserved in the tetrameric molecule.

Hematological investigation of a 6-week-old infant with severe hemolytic anemia revealed a decreased hemoglobin concentration of 53 g/L, a decreased hematocrit of 0.15, and a decreased erythrocyte count of 1.8 × 10^{12}/L. In addition the infant had an increase in reticulocytes (322 × 10^{11}/L) and bilirubin (96 μmol/L). Blood films showed a normochromic picture with irregularly shaped cells, elliptocytes, and erythrocyte fragments suggestive of an erythrocyte membrane defect. G6PD and pyruvate kinase concentrations were within reference intervals, and a presumptive diagnosis of hereditary elliptocytosis was made.

Cellulose acetate and citrate agar electrophoresis results were both within reference intervals, as was cation exchange chromatography on the Bio-Rad Variant β-thalassemia system. Examination of whole lysate by electrospray ionization mass spectrometry on a VG Platform (1) showed that the complement of β (15 867 Da), γ^C (15 995 Da), and γ^A (16 010 Da) chains were within reference intervals for the age of the infant. However, the valley between the Na and K adducts of the α chain (15 126 Da) contained a new component with a mass increase of 30–33 Da over the normal α chain (Fig. 1A). Reversed-phase HPLC on a C-4 Jupiter column (2) failed to separate out the new component, so DNA sequencing was used to identify the putative mutation.

The entire coding regions of both the hemoglobin alpha 1 and alpha 2 genes were individually amplified from genomic DNA using the primer pairs 5′-TGG AGG GTG GAG ACG TCC TG-3′ with 5′-CCA TGC TGG CAC GTT TCT GA-3′, and 5′-TGG AGG GTG GAG ACG TCC TG-3′ with 5′-CCA TTG TTG GCA CAT TCC GG-3′, respectively, and sequenced on an ABI 3130xl genetic analyzer with Big Dye Terminator v3.1 cycle sequencing chemistry according to the manufacturer’s recommendations. This process revealed heterozygosity for a GCC→ACC transition at codon 82 of the α1 gene, and we have named this new α82Ala→Thr variant Hb Hagley Park.

Unstable hemoglobinopathies are a well-recognized cause of hemolytic anemia, and the identification of this novel substitution raises the question as to whether it contributed to the hemolytic condition. This question could be answered by stability tests; however, insufficient blood (50 μL) was available for analysis and, in any case, the presence of substantial amounts of HbF, which registers a positive result in these tests, would confound the results. Family studies would also be expected to be informative; however, neither parent was available for analysis.

There are no published reports of any other mutation at position α82, the 3rd residue of the F helix. This short amphipathic helix contains the proximal histidine, and its inner hydrophobic surface makes direct contact with the heme plate. Mutations along this surface result in molecular instability and can cause mild hemolytic anemia (1–3). The Ala82 side chain is positioned between the inner and outer hydrophilic surface of the helix, and introduction of an additional CH_2OH moiety could potentially destabilize the structure. However, alignment of 439 α-globin sequences from different species shows that residue α82 is only loosely conserved as alanine, and some 18 species, including mouse and rabbit, have a threonine residue at position α82. Thrreonine is also found at the F3 position of the human β chain, supporting the notion that its occurrence in the α chain should not cause any major distortion, at least of the helix.