Hb E1c as an Indicator for the Presence of Hb AE Phenotype in Diabetic Patients, Shiu Chuen Wong* and Tar Choon Aw (Dept. of Lab. Med., National Univ. Hosp., 3rd Flr., 5 Lower Kent Ridge Rd., Singapore 117059; *author for correspondence: fax 65-777-1613)

Since the observation of a “fast-moving” hemoglobin (Hb) in diabetic blood specimens by Rahbar in 1968 [1] and the subsequent structural identification of the glucose-“modified” Hb [2], the measurement of erythrocyte glycoHb (Hb A1c) has served as the monitor for long-term glucose control for patients with diabetes mellitus [3]. Column chromatography was one of the first methodologies used for the quantification of Hb A1c [4]. Recent modifications of the methodology, including shorter column size and faster turnaround time, have resulted in the application of automated HPLC for Hb A1c analysis. As column chromatography has always been a major tool for the investigation of human Hb variants, the use of automated HPLC systems for the analysis of Hb A1c in clinical laboratories renders an extra opportunity for detecting abnormal Hbs in clinical blood specimens, e.g., Hb Manitoba, Hb G-Coushatta, Hb Turriff, and Hb Sherwood Forest [5–7]. The presence of an abnormal Hb will result in the formation of its own minor glycoHb; the total glycoHb in the red cells of a Hb variant trait carrier is then the sum of the glycoHb A and the glycoHb variant. For example, the total glycoHb in a sickle cell trait carrier (Hb AS) is Hb A1c + Hb S1c. We describe in this report the chromatographic properties of the minor glycoHb E (α2β226Glu→Lys) in an automated HPLC system, and the usefulness of the detection of the Hb E1c peak in the HPLC chromatogram as the indicator for the presence of Hb E in the patient.

This study involved EDTA whole-blood specimens specifically for Hb A1c analysis. Hospital in-house specimens arrived in the authors’ NUH Referral Laboratories within 2 h, whereas referral samples were delivered overnight by courier. The general protocol was: (a) Hb A1c assay by HPLC, and (b) hemoglobinopathy studies on specimens whose HPLC chromatograms suggested the presence of abnormal Hb peaks. Hb A1c assay of the red cell hemolysates of the specimens was carried out with an automated Diamat HPLC system (BioRad), which involved a spherical cation-exchange gel column and a 6.5-min step-gradient created by a single-piston pump and three phosphate buffers of increasing ionic concentration. Chromatography was carried out at 10 °C, and the elution of Hb fractions was monitored at 415 nm and 690 nm. A built-in integrator performed the data analysis. Hemoglobinopathy studies were carried out according to established procedures, including: (a) the initial detection and identification of abnormal Hbs by alkaline and acid electrophoreses on Helena’s cellulose acetate and citrate agar plates, and (b) the quantification of Hb fractions (Hb A1c, F, A2, and E) in red cell hemolysates by a Variant cation-exchange HPLC system (BioRad) programmed with a 6.5-min gradient. Both the Diamat and Variant systems had previously been evaluated in the authors’ laboratory; the latter elutes Hb E and Hb A2 as a single major peak [8, 9]. Criteria for designating Hb AE phenotype to a blood specimen were: (a) the presence of a slow-moving Hb variant in the Hb E/Hb A2 position on alkaline cellulose acetate gel, (b) one single Hb A/Hb E band on acid agar plate, (c) a major Hb E/Hb A2 peak at the end portion of the Variant HPLC chromatogram, and (d) positive test for unstable Hb by the isopropanol assay [10].

A total of 3144 Hb A1c blood specimens (48% NUH inpatients, 47% local outpatients, 5% others) was analyzed in the first 6 months of this year. Fig. 1A is a Hb AA Diamat HPLC chromatogram, showing a minor Hb A1c peak eluted at 2.8 min and a major Hb A peak at 4.0 min. Fig. 1B is the Diamat HPLC chromatogram on another specimen, where an asymmetrical Hb A1c peak with a “right shoulder” was observed. Hemoglobinopathy studies on this specimen revealed the presence of a slow-moving Hb variant, most likely Hb E, with the following properties: electrophoretic mobility in the E/A2 position on alkaline cellulose acetate gel, identical electrophoretic mobility as Hb A on acid citrate gel, coelution with Hb A2 on cation-exchange chromatography, and positive isopropanol unstable Hb test.

During a 6-month period, nine Hb A1c blood specimens were found to have the asymmetrical Hb A1c peak with a “right shoulder,” all these specimens were confirmed by hemoglobinopathy studies to have the phenotype of Hb AE. The ethnic origins of the nine Hb E trait carriers were: six Malays, two East Indians, and one Indonesian. Their hematological data were: RBC = 4.4 × 1012/L (3.91–5.91); Hb = 109 g/L (97–138); mean cell volume (MCV) = 77.7 fl (68.9–86.1); mean cell Hb (MCH) = 24.6 pg (21.1–27.4); mean cell Hb concentration (MCHC) = 317 g/L (298–340). The average percentage of Hb (E+A2) in seven of the nine samples (as quantified by the Variant HPLC) was 28.3% (25.5–31.0%).

The “right shoulder” peak had an elution time of 3.0–3.1 min. Its identity was inferred from the analysis of a patient who had been diagnosed to be Hb EE (or Hb E-b-thalassemia), with only Hb E and no Hb A, MCV = 65.1 fl, and MCH = 23.1 pg. Fig. 1C shows the Hb EE chromatogram: 2.8% Hb F (at 2.0 min), 4.2% minor peak (at 3.0 min), and 92.6% Hb E (at 4 min). The 4.2% minor Hb eluted at 3.0 min was Hb E1c, as Hb E was the only major Hb in this sample. Thus, it could be inferred that the Hb E1c in the Hb AE blood specimens was eluted as the “right shoulder” in the chromatogram, since the elution time of Hb E1c (3.0 min) was slightly behind that of Hb A1c (2.8 min). No similar “right shoulder” was observed in the analysis of red cell hemolysates from trait carriers of Hb S (α2β226Glu→Val) and Hb D (α2β2121Glu→Gln).

The chromatographic properties of the red cell Hbs from diabetic individuals have been well studied [11]; those of Hb E can be found in a review by Huisman [12]. Cation-exchange HPLC with a SynChropak CM300 column and a sodium acetate gradient resolves Hb E1c completely behind the Hb F fractions (F1 and F2) but in front of the other major Hbs (A and E). Hb E is well known to coelute with the normal minor Hb A2 (α2δ2) on both anion and cation exchangers (unless a
very slow gradient on a Baker-Bond PEI-WAX column is used). This presents a problem for the accurate quantification of Hb E, especially in cases of \(\beta\)-thalassemia trait carriers with increased Hb A2. Nevertheless, the fact that Hb E resolves completely from Hb A in both anion and cation exchangers renders column chromatography an invaluable tool for the screening of Hb E in both adults and newborns \([13, 14]\). The Diamat HPLC system, similar to the SynChropak CM300 column, resolves the glycosylated minor Hbs between the Hb F fractions and the other major Hb fractions. However, the built-in short programs in these commercial “Hb A1c automated HPLC analyzers” coelute Hb E (and other common variants Hb S, Hb D, Hb C) with Hb A as one single Hb peak at the end of the chromatogram, making it impossible to detect the presence of Hb E in any Hb AE red cell hemolysates.

This study demonstrates that the extra peak detectable as the “right shoulder” of the Hb A1c peak is most likely Hb E1c, when blood specimens are analyzed fresh within 24 h. Results of the follow-up hemoglobinopathy studies confirm that the presence of the Hb E1c “right shoulder” peak in the chromatograms of fresh red cell hemolysates can serve as an indicator for the presence of Hb AE phenotype in the patient. In practice, the presence of Hb E1c will affect the integration of the Hb A1c percentage: (a) The total glycoHb in Hb AE specimens is the sum of Hb A1c + Hb E1c; (b) the presence of the extra “right shoulder” Hb E1c peak may increase the interpeak trough between the Hb components in the chromatogram, resulting in a reduction in the integration of the total Hb A1c + Hb E1c percentage \([15]\). Thus, one should note that, for the purpose of monitoring the long-term control of diabetic patients, the changes in either percent Hb A1c or percent total glycoHb, between clinic visits, will provide the relevant information, provided the reports are consistent in regard to methodology and calculation.

References
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Selective Screening for Hyperhomocysteinemia in Pediatric Patients. M. Antònia Vilaseca,1,5 Dolores Moyano,4 Rafael Artuch,3 Imma Ferrer,1 Mercè Pineda,2 Esther Cardo,2 Jaume Campistol,2 Carles Pavia,3 and José-Antonio Camacho3 (Serv. de 1Bioquím., 2Neurol., 3Pediatr., Hosp. Univ. Sant Joan de Déu, Passeig de Sant Joan de Déu 2, 08950 Esplugues, Barcelona, Spain; 4author for correspondence: fax 34-3-2803626)

Hyperhomocysteinemia is a common condition with genetic and environmental causes [1], and associated, especially in its severe form, with risk of premature atherosclerosis and thrombosis [2]. Testing for hyperhomocysteinemia in children may be useful for investigation of patients with clinical features of homocystinuria [2], and for the assessment of nutritional status [3]. Moreover, the investigation of causes of mild hyperhomocysteinemia, such as diabetes [1] or renal failure [4], may clarify the etiology of this abnormality. The possibility of easily correcting hyperhomocysteinemia by diet suggests potential utility of screening within some populations [5].

Screening for hyperhomocysteinemia in children has rarely been reported. In infants, total homocysteine (tHcy) measurement was reported only in a group on macrobiotic diets [6] and, in children, in a group with leukemia [7]. Recently, a group of children with premature cardiovascular death in their male relatives was studied and the modest increase of plasma tHcy was partly attributed to genetic factors [8].

Our aim was to screen for hyperhomocysteinemia in children at risk, owing to genetic or environmental causes, (a) to discover individual patients with primary hyperhomocysteinemia and (b) to compare tHcy concentrations of certain groups of children at risk with those of age-matched reference values.

Screening was performed in the following groups: Group 1: genetic factors: (a) possible heterozygotes for cystathionine β-synthase (CBS; EC 4.2.1.22) deficiency (n = 10) among young relatives of patients; (b) Marfan phenotype (n = 5); (c) stroke (n = 53): ischemic infarct and (or) thrombosis (n = 38); hemorrhage (n = 13) and MELAS syndrome (n = 2); (d) megaloblastic anemia (n = 1); Group 2: secondary abnormalities: (a) well-controlled diabetes mellitus without renal failure (n = 135); (b) renal failure (n = 13); nephrotic syndrome (n = 7), cistinuria–lysinuria (n = 2) and Fanconi syndrome: cystinosis (n = 1), Löwe syndrome (n = 2), and tyrosinemia type I (n = 1), and (c) anorexia nervosa (n = 43).

Reference values were established in apparently healthy children who underwent presurgical analysis for minor interventions (n = 195; age range 2 months–18 years). tHcy was independent of sex, and increased significantly with age [9] (Table 1). Samples of patients and controls were obtained in accordance with the Helsinki Declaration of 1975, as revised in 1983.

tHcy was determined by HPLC with fluorescence detection [9].

We found (Table 1) two patients with primary CBS deficiency: a 12-year-old girl with marfanoid phenotype and cataracts and a 6-month-old male with fatal hemorrhagic infarct. Moreover, we found a patient with a probable defect of cobalamin metabolism (CblE or CblG), with isolated homocystinuria associated with megaloblastic anemia. We also found 7 of 10 probable heterozygotes for CBS deficiency with mild hyperhomocysteinemia. Significant differences were found in children with stroke (P < 0.02–P < 0.0001) and in adolescents with anorexia nervosa (P < 0.001–P < 0.0001). In the stroke group, tHcy did not show significant differences in children with hemorrhage (median 7.5; range 2.6–25.2 μmol/L; n = 13) compared with those with ischemic infarct (median 7.7; range 3.5–15 μmol/L; n = 38), so that all of them were grouped only according to age (Table 1). Three patients from this group showed mild hyperhomocysteinemia (15, 16.5, and 25.2 μmol/L) of undetermined etiology. Children with renal failure and diabetes mellitus did not show significant differences compared with reference values.

The use of very sensitive methods with low detection limits (HPLC with fluorescence detection) to screen for tHcy is necessary, because moderate hyperhomocysteinemia (15–40 μmol/L) may be difficult to detect by routine amino acid analysis in some genetic defects [such as vitamin B12-responsive CBS, 5,10-methylenetetrahydrofolate reductase (EC 1.7.99.5), or cobalamin metabolism defects] and undernutrition states [5]. Even with sensitive methods, detection of heterozygotes for CBS deficiency is poor in basal conditions and after methionine loading [10]. Reference values for the loading test are difficult to obtain in children for ethical reasons. However, the investigation of family members of homocystinuric patients either by tHcy measurement or by molecular analysis of known mutations seems necessary in view of the risk involved in mild lifelong hyperhomocysteinemia and the possibility of correcting it by vitamin supplementation [1].

An increasing number of young patients have recently been reported with stroke episodes caused by genetic defects in homocysteine metabolism, either isolated or associated with other risk factors of thrombosis (factor V Leiden, protein C or S or antithrombin III deficiency) or exogenous triggering factors (diarrhea, dehydration, surgery) [11, 12]. We diagnosed a classic homocystinuria