An Automated Hb A_2 Analyzer, HLC-723G7, for Diagnosis of β-Thalassemia

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ABSTRACT. We investigated the usefulness of an automated analyzer, the HLC-723G7, for determination of Hb A_2 in the diagnosis of β-thalassemia. The analyzer was used for 250 Thai subjects who were either normal or were diagnosed with microcytic hypochromia based on hematological findings. An intra-assay precision test of normal subject (Hb A_2=2.78%) and that (Hb A_2=5.90%) with β-thalassemia revealed as coefficients of variation (CV%) of 3.2% and 0.95%, respectively. Samples were kept stable at 4°C for at least 10 days. The Hb A_2 values obtained by the HLC-723G7 showed good correlation with those obtained by DEAE-HPLC with the coefficient of correlation (r) being 0.940, the coefficient of determination (R^2) 0.884, and the equation of the regression line $Y_{G7}=1.057X_{DEAE}-0.248$. The subjects with a higher Hb A_2 value than about 4% were suspected of having β-thalassemia based on hematological findings. Most of those with a lower or relatively lower Hb A_2 value than the normal range (2.3-3.5%) or 4% and hematological abnormalities were considered to be suffering from α-thalassemia-1, α-thalassemia-2 or iron deficiency anemia. However, a confirmed diagnosis must be made by the further study of the red cell morphology, clinical manifestations and by the DNA analysis.

Key words : Automated Hb A_2 analyzer, HLC-723G7 — Hemoglobin A_2 (Hb A_2) — β-Thalassemia — α-Thalassemia — Thalassemia diagnosis

Measurement of Hb A_2 and Hb F, which are minor components of human Hb, is useful for the differential diagnosis of several anemias and thalassemias (thal). Specifically, an increased Hb A_2 value is considered to be a β-thal trait.[1] In the past, Hb A_2 measurement has been done either by eluting Hb bands from cellulose acetate membrane following electrophoresis or by anion exchange chromatography (micro-column method).[2] Recently, high performance liquid chromatography (HPLC) on an anion exchange resin column has increasingly been used with quite good reproducible results and savings in time.[3-5] The aim of this study was to

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examine the possibility of using a commercially available automated analyzer, the HLC-723G7(G7), for the measurement of Hb A_2 in the diagnosis of β-thal. HPLC with an anion exchange resin column (DEAE),[6] which has been routinely employed for Hb A_2 quantification in our laboratory, was used for comparison with it.

SUBJECT

The 250 Thai subjects excluded the carrier of Hb variant recruited from the Hb electrophoresis laboratory were 66 males and 184 females, aged 7 to 83, with mean age of 29.8 years. Fifty of these subjects were normal (MCV > ca. 85 fL, MCH > ca. 29 pg) while the hematological findings of the other 200 subjects indicated that they had microcytic hypochromia.

MATERIALS AND METHODS

The red cells were separated from plasma by centrifugation and frozen for transport to the Department of Biochemistry, Kawasaki Medical School, Kurashiki, Japan. A 25 μL aliquot of red cells was added to 50 μL of distilled water and 30 μL of carbon teterachloride, mixed well by vortex and centrifuged to collect the clear supernatant as the hemolysate. Since the hemolysate might include metHb and aged or oxidized Hb, a 50 μL aliquot of the hemolysate was treated with 1 μL of 5% KCN solution and 1 μL of 0.1 mol/L dithiothreitol (DTT) solution before analysis of the Hb components. Measurement of Hb A_2 by the automated Hb A_2 analyzer, the G7, with a cation exchange resion column (Tosoh Corporation, Tokyo, Japan) was performed using to the protocol recommended by the manufacturer.[7] HPLC with an anion exchange resin column (DEAE-5PW 7.5 × 75 mm, Tosoh Corporation, Tokyo, Japan),[6] in which the elution solution used was a linear gradient buffer containing 20 mmol/L Tris-HCl (pH 8.25) mixing with 0.5 mol/L NaCl-20 mmol/L Tris-HCl (pH 8.0) (0% →30%) for 20 minutes at a flow rate of 1.0 mL/min at room temperature and detection at 415 nm, was compared with the results from G7. The time required for analysis of one sample by DEAE was at least 30 minutes including column clearance, while for the G7 it was within eight minutes. A statistical analysis of the results was performed using StatView Analytical Software (SAS Institute Inc. Cary, NC, USA).

RESULTS

Although the DEAE elution pattern routinely used in our laboratory, as shown in Fig 1, and that of the hemolysate prepared from red cells transported from Thailand, as seen Fig 2, seems to include metHb or oxidized Hbs, use of a hemolysate treated with KCN and DTT decreases the contents of those extra Hb peaks and is considered to achieve the most accurate Hb A_2 quantification. Therefore, the Hb A_2 quantification using red cells from Thailand in this study was performed with a hemolysate treated with KCN and DTT.

Fig 3 shows the elution pattern of the Hbs determined by the G7. The first zone included the Hb F fraction at a retention time around 0.9 min

from the starting point. The Hb A fraction was eluted at a retention time around 3.2 min. The Hb A_2 fraction eluted after Hb A overlapped the foot of the Hb A peak. The quantification of these Hb fractions was achieved by estimating the size of each area of the Hb total area. If there are abnormal Hbs, e.g. Hb S and Hb E, they appeared as peaks at

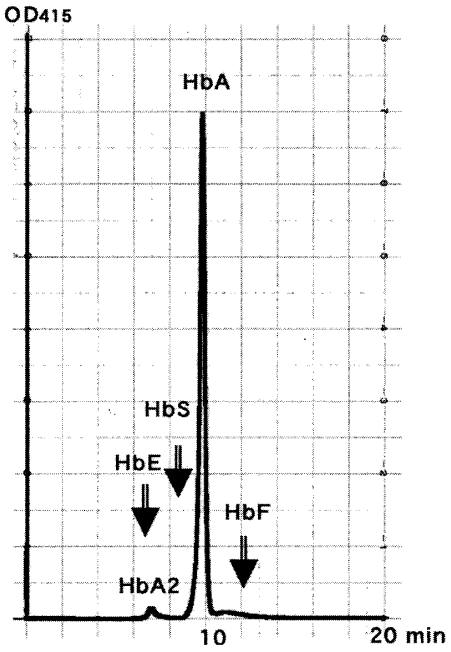

Fig 1. Chromatogram of the fresh hemolysate analyzed by DEAE-HPLC. The positions eluted, Hb E and Hb S, are indicated by arrows.

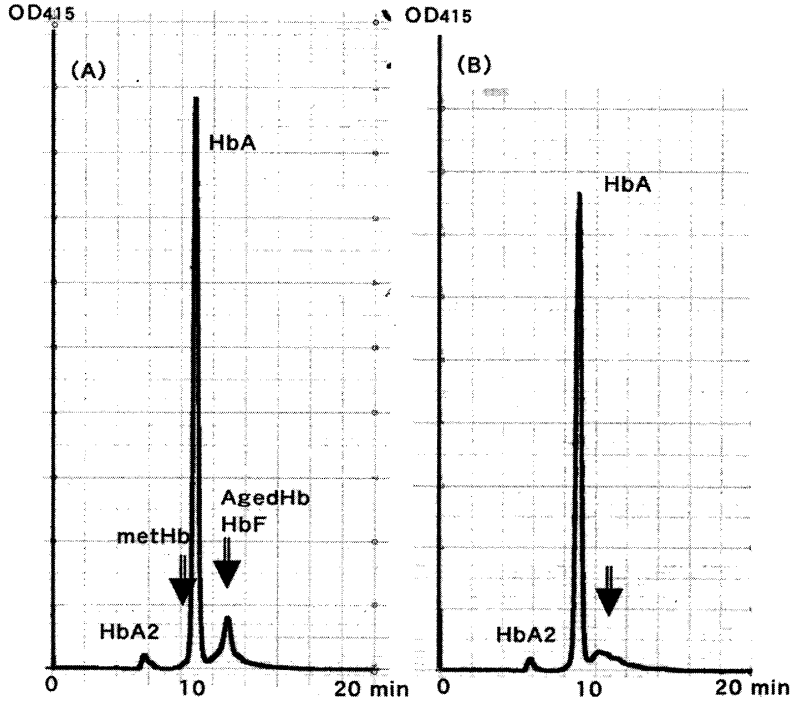

Fig 2. The comparison of the elution pattern of the hemolysate prepared from red cells sent from Thailand with one untreated (A) and treated (B) with KCN and DTT.

positions depending on their ionic properties or interaction between the Hb and resin. As shown in Fig 3, Hb E superimposed at the same retention time as Hb A_2. As a result, accurate quantification of the Hb A_2 of the Hb E carrier may be impossible to estimate. Since Hb S is revealed at a longer retention time than Hb A, there is not interference with Hb A_2 quantification. In the elution pattern of Hbs by DEAE, as shown in Fig 1, on the other hand, the first elute is the Hb A_2 fraction, followed by Hb A and, then, Hb F and aged or oxidized Hb. The Hb S and Hb E fractions are eluted at the positions indicated in Fig 1. The Hb A_2 fraction is also difficult to separate from Hb E.

The intra-assay precision of the G7 analyzer was determined using samples from two subjects with different concentrations of Hb A_2. The intra-assay precision was estimated with a single batch at concentrations of 2.78% and 5.90%. The coefficients of variation (CVs) within the batch were 3.2% and 0.95%, respectively (Table 1).

The stability of the hemolysate treated with KCN and DTT was maintained at 4°C for at least 10 days.

Correlation of the Hb A_2 values of the 250 subjects obtained by the G7 and DEAE methods was good, as shown in Fig 4. The coefficient of correlation (r) was 0.940, the coefficient of determination (R^2) was 0.884 and the equation of the regression line was $Y_{G7}=1.057X_{DEAE}-0.248$. In Fig 4, a subject with the Hb A_2 value higher than about 4% by DEAE was considered to have microcytic hypochromia based on hematological findings (CBC). The subject with a normal value of Hb A_2 or less than 4% were either normal or had microcytic hypochromia (Fig 5). Subjects with a high Hb A_2 value might be diagnosed as having a β-thal trait. Those with a

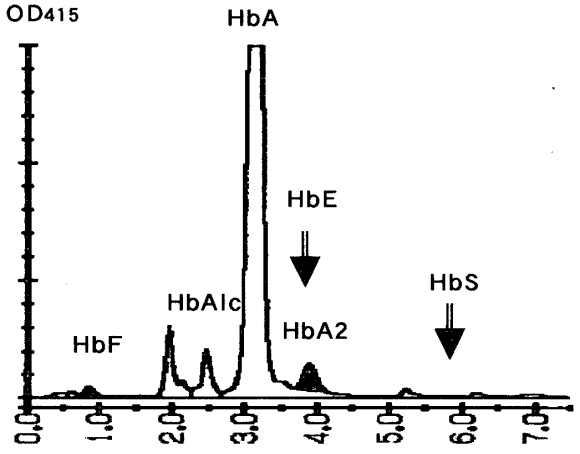

Fig 3. Chromatogram of Hb analyzed by the Automated Hb A_2 Analyzer, HLC-723G7. The elution positions of Hb E and Hb S are indicated by arrows.

TABLE 1. Precision test (n=10) of the Hb A_2 value using hemolysates with normal or β-thalassemia determined by the HLC-723G7.

	Means	SD	CV%
Normal	2.78	0.089	3.2%
β-thalassemia	5.90	0.056	0.95%

lower Hb A_2 value might be diagnosed as normal or might be silent (α-thal-2) or mild (α-thal-1) α-thal based on clinical and CBC findings. To the subjects suspected of α-thal or β-thal, their DNA will be further investigated in detail and decided absence or presence of some gene abnormalities.

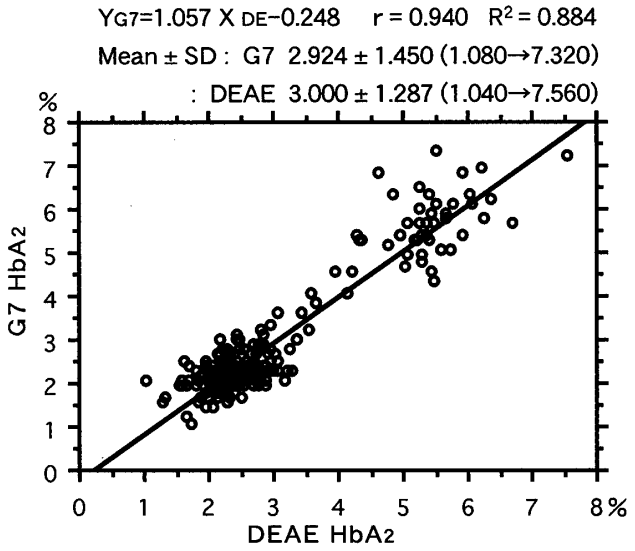

Fig 4. Correlation analysis of the Hb A_2 values measured with the HLC-723G7 and by DEAE-HPLC. The hemolysates used here were treated with KCN and DTT before measurement. The mean and standard deviation of Hb A_2 of the all subjects by HLC-723G7 and DEAE-HPLC were 2.924 and 1.450 (1.080→7.320), and 3.000 and 1.287 (1.040→7.560), respectively. Based on this correlation analysis, r=0.940, R^2= 0.884 and regression line Y=1.057X−0.248.

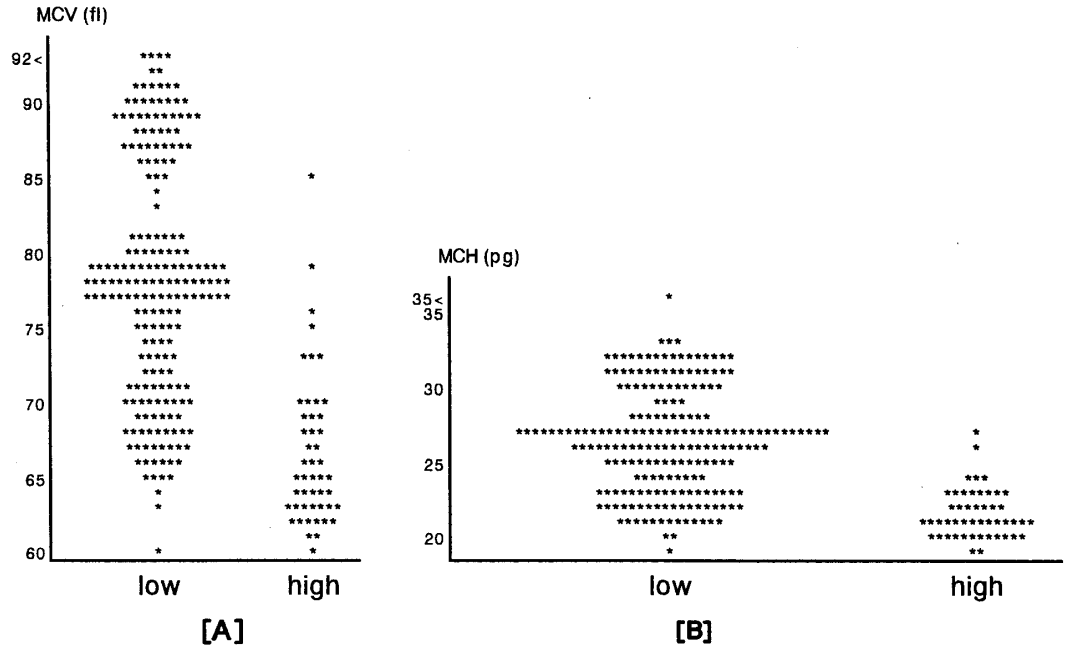

Fig 5. Distribution of MCV [A] and MCH [B] values in the subjects with lower Hb A_2 (<4%) [low] or higher Hb A_2 (>4%) [high] values. Many subjects having lower MCV (<80 fL) or MCH (<25 pg) values are included among the subjects having Hb A_2 value lower than 4%.

DISCUSSION

The Hb component of the normal adults consists of approximately 96% Hb A ($\alpha_2\beta_2$) as the main Hb, and 2-3% of Hb A_2 ($\alpha_2\delta_2$) and about 1% Hb F ($\alpha_2\gamma_2$) as minor components. The production of these Hbs is controlled by structural genes, the β-globin-like gene cluster, 5'-ε-$^{G}\gamma$-$^{A}\gamma$-$\psi\beta$-δ-β-3', on the short arm of the 11th chromosome and the α-globin-like gene cluster, 5'-ζ-$\psi\zeta$-$\psi\alpha 2$-$\psi\alpha 1$-$\alpha 2$-$\alpha 1$-ϑ-3', on the short arm of the 16th chromosome. Thal is a group of genetic disorders characterized by microcytic hypochromia with absence or reduction of α- or β-globin chain synthesis of the Hb molecule. In α-thal, there is absence or reduction of α-globin synthesis of the Hb molecule, while in β-thal β-globin chain synthesis is absent or reduced. In the case of β-thal, the presence of the affected β-globin gene seems to cause, at glance, a relative increase in the activity of the δ-globin gene or the γ-globin gene in comparison with that of the β-globin gene as well as an increase in the content of Hb A_2 or Hb F.[8-10] There are, of course, also γ-thal, δ-thal and ζ-thal due to the corresponding gene defect, but these thals are not important in human adults, since the corresponding Hb contents are relatively lower in the total Hb.[11-14] It is very interesting in that β-thal presents clinically with severer symptoms and reveals obvious abnormalities in hematology, red cell morphology and the content of minor Hbs, especially the Hb A_2 value. Therefore, we have been attracted to a newly improved method for quantifying precise Hb A_2 values for the diagnosis of β-thal. Until now, we have used DEAE method for this purpose. Since they are considered to be more precise analytical instruments than the cellulose acetate membrane electrophoresis-elution method or micro-column chromatography, which have been recommended as standard methods by the International Hematological Society.[2] However, since neither the G7 or the DEAE methods nor the standard methods can separate Hb E from Hb A_2, they are not good enough for use in the diagnosis of β-thal in patients living in areas where Hb E is prevent (Fig 1 and 2). Otherwise, the G7 method can automatically quantify the Hb A_2 value in a sample within 8 min and shows good results as a precision test (Table 1). It is considered to be a good instrument for the screening of β-thal (Fig 3). Among the subjects investigated here, there were many subjects with a Hb A_2 value higher than about 4% and with microcytic hypochromia in their CBC. These can be easily diagnosed as a β-thal trait (Fig 4 and 5). Additionally, there were many subjects with a lower or relatively lower Hb A_2 value than the normal range who were suspected to have various types of α-thal based on their CBC. α-Thals, not only the deletion types of α-thal-2 ; e.g. $-\alpha^{3.7}$ and $-\alpha^{4.2}$, but the deletion types of α-thal-1 ; e.g. --SEA and --Med, are often found worldwide[15,16] under similar conditions to those in Thailand. These might possibly be detected in subjects having a lower Hb A_2 value and microcytic hypochromia in their CBC.[17] It is also worth noting that there is an iron deficiency anemia condition similar to those of the thals in its hematology, red cell morphology and Hb A_2 value.[18,19]

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