THE VALIDATION OF AN AUTOMATED LIQUID CHROMATOGRAPHY SYSTEM FOR THE DIAGNOSIS OF THALASSEMIAS AND HEMOGLOBINOPATHIES

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Abstract. Accurate and precise hemoglobin separation and the quantitation of Hb A_2 and Hb F are essential for the diagnosis of the thalassemias and hemoglobinopathies. Presented in this study is the validation of the the Hb A_2 assay of the HbGold analyzer, a fully automated liquid chromatography system for hemoglobin separation and quantitation. Variability of Hb A_2 quantitation was quite low; the CV's of within-run, between-run and interlaboratory studies were 1.8-3.1%, 3.4-6.0% and 6.8-8.8% respectively. The results of the %Hb A_2 quantitated by HbGold analyzer correlated well with those given by the Bio-Rad Variant Hb testing system (r=0.98). The application of the HbGold analyzer for the diagnosis of the thalassemia phenotypes frequently observed in Thailand is considered. In conclusion, the Hb A_2 assay of the HbGold analyzer could be used for the quantitation of Hb A_2 and Hb F and the presumptive identification of abnormal hemoglobins.

INTRODUCTION

The thalassemias and hemoglobinopathies are the most common inherited human diseases. It is estimated by the WHO that 4.5% of the world's population, some 250 million people, carry a potentially pathological gene; 300,000 infants are born with major hemoglobin disorders every year (WHO, 1999). Increasing global migration has introduced the diseases into many areas where they were not originally endemic. Thalassemias are characterized by defective synthesis of one or more of globin subunits of the hemoglobin molecule. Thalassemias can be subdivided, on genetic principles, into 5 groups, including α-thalassemia, β-thalassemia, δβ-thalassemia, δ-thalassemia and γδβ-thalassemia. The important groups found in Thailand are α- and β-thalassemia. In α-thalassemia a reduction of α-globin chain leads to an excess of γ- and β-globin chains which form the homotetramers, Hb Bart's (γ_4) and Hb H (β_4) respectively. The presence of Hb Bart's or Hb H is the hallmark of α-thalassemia. β-thalassemia results from a reduction of production of the β-globin chain. Elevation of Hb A_2 is the most important feature in identifying heterozygous β-thalassemia. Homozygous β-thalassemia is associated with a predominance of Hb F and variable amounts of Hb A_2. Hemoglobinopathies or structural hemoglobin variants may be due to the substitution of one amino acid for another, the deletion of a portion of the amino acid sequence, the abnormal hybridization of two chains, or the abnormal elongation of the globin chain. The abnormal chains may be alpha, beta, gamma, or delta chains. To date, approximately 750 hemoglobin variants have been reported (Huisman *et al*, 1998). In Thailand, HbE and Hb Constant Spring are the two most common abnormal hemoglobins (Wasi *et al*, 1980).

The 1975 International Committee for Standardization in Hematology Expert Panel

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on Abnormal Hemoglobins and Thalassemias made recommendations for the laboratory investigation of these conditions. Recommended initial tests include a complete blood count (CBC), hemoglobin electrophoresis on cellulose acetate at alkaline pH and quantitation of Hb A_2 and Hb F. Conventional methods for quantitation of Hb A_2 include anion-exchange column chromatography and cellulose acetate electrophoresis with elution and absorptive spectrophotometry (Steinberg and Adams, 1991; WHO, 1994; BCSH, 1998; Clarke and Higgins, 2000). Conventional methods for quantitation of Hb F include radial immunodiffusion (RID) and alkali denaturation (BCSH, 1998; Clarke and Higgins, 2000). Although these conventional methods are reproducible and accurate, they are labor-intensive and time-consuming. High performance liquid chromatography (HPLC) has been established as an accurate method for hemoglobin separation and determination although it is relatively expensive (Huisman, 1987; WHO, 1994).

The HbGold analyzer is a fully automated system that uses cation exchange chromatography in conjunction with gradient elution to separate and quantitate area percentages for Hb A_2, Hb F and other abnormal hemoglobins. Presented in this study is the validation of the Hb A_2 assay of the HbGold analyzer for diagnosis of thalassemias and hemoglobinopathies. Performance characteristics, including precision and accuracy were studied. The β-thalassemia short program of the Bio-Rad Variant hemoglobin testing system, whose results are known to be correct through comparative studies with a definitive method (Fucharoen *et al*, 1998), was used as the reference method in a comparison of methods experiment. Application of the Hb A_2 variant assay of the HbGold analyzer was studied in subjects who attended the Thalassemia Clinic, Department of Pediatrics, Faculty of Medicine, Siriraj Hospital, Mahidol University, Thailand.

MATERIALS AND METHODS

Hemoglobin separation and quantitation by the Hb A_2 assay of the HbGold analyzer

Hemoglobin separation and determination by the Hb A_2 assay of the HbGold analyzer (Drew Scientific Limited, Cumbria, United Kingdom) were performed according to the manufacturer's instructions. Ten microliters of EDTA blood was diluted with 1.0 ml of de-ionized water. Each sample vial was covered with parafilm, mixed by vortex mixer, and placed in the 100-position autosampler.

The HbGold analyzer comprises a pair of syringe pumps, three multiport rotary valves, and a fixed wavelength flow-through spectrophotometer in conjunction with a computer control system to perform cation exchange chromatography. Sample loading from the autosampler is achieved by using one of the syringe pumps to aspirate the sample from the sample vial into the loading loop situated on the rotary valves. The rotary valves allow the syringe pumps to fill from reagent bottles and then reverse direction to deliver a flow of reagent to the chromatography column. The gradient of increasing eluent strength is produced by varying the relative flow rates of the two syringe pumps. The hemoglobin fractions are eluted in order of the ionic strength and detected by a transmission detector at 415 nm; they are identified by their characteristic retention time; the result is shown graphically in real time on a video screen. At the end of the elution cycle the amount of each hemoglobin fraction is expressed as a percentage of the total hemoglobin concentration. The HbGold analyzer can store both chromatogram and report data from 100 sample results. The stored sample results may be printed, deleted, backed-up or transmitted to a laboratory computer; stored results can be recalled and recalculated.

A de-ionized water sample is run as a blank chromatogram at the beginning of each batch: the HbGold analyzer subtracts this background from each of the actual sample chromatograms. In addition, peak tracking is used to accommodate slight changes in peak retention time which occur during the reagent lifetime. The peak tracking sample is prepared in the same way as the rest of the samples

to be analyzed. It should be analyzed as soon as possible after preparation to reduce the possibility of interference from degradation products.

Hemoglobin separation and determination by the β-thalassemia short program of the Variant hemoglobin testing system

Hemoglobin separation and determination using the β-thalassemia short program of the Variant hemoglobin testing system were performed according to Bio-Rad's instructions (Bio-Rad Laboratories, California, USA); the test method was that described by Fucharoen *et al* (1998). In this study, the Variant hemoglobin testing system was used as the reference method in the comparison of methods experiment.

DNA analysis

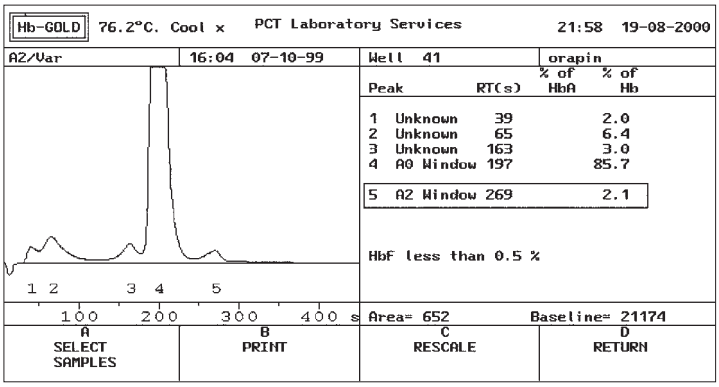
The presence of β-thalassemia and Hb E was confirmed by reverse dot blot (RDB) hybridization between the amplified DNA and the allele-specific oligonucleotide probes (Winichagoon *et al*, 1999). Detection of α-thalassemia 1 was performed by gap PCR, as described by Winichagoon *et al* (1995).

Statistical analysis

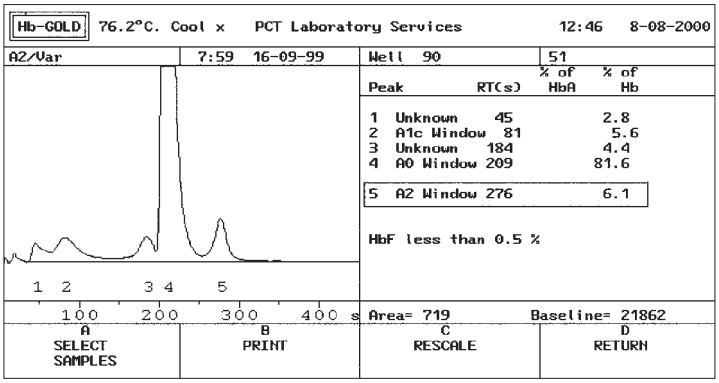
Mean, standard deviation (SD) and coefficient of variation (%CV) were used to estimate the degree of imprecision. Correlation of % Hb A_2 measured by the HbGold analyzer and Variant hemoglobin testing system were determined by linear regression analysis using each value as a variable. The strength of association between the two variables was measured by the correlation coefficient.

RESULTS

The Hb A_2 assay of the HbGold analyzer could be used to separate and quantitate Hb A_2, Hb F and other abnormal hemoglobins. They were quantified as a percentage of total hemoglobin. Fig 1A shows the chromatogram of a normal subject with Hb A_2 and Hb A. The retention times of Hb A_2 and Hb A were 275-290 and 170-235 seconds respectively; the total analysis time was 6.5 minutes. In β-thalassemia trait (Fig 1B), the chromatogram was similar to that of the normal subject, but the percentage of Hb A_2 was increased.



(A)



(B)

Fig 1–Chromatograms of normal subject (A) and β-thalassemia trait (B).

Performance characteristics of the Hb A_2 assay of the HbGold analyzer

Precision: Two measures of precision including repeatability and reproducibility were made in order to assess the variability of Hb A_2 quantitation.

1. **Repeatability.** Repeatability was performed under constant conditions in the He-

Table 1
Within-run and between-run variability of %Hb A_2 by the HbA_2 assay of the HbGold analyzer.

Sample types	N	Within-run			Between-run		
		Mean	SD	%CV	Mean	SD	%CV
Control I	20	2.0	0.06	3.0	2.0	0.12	6.0
Control II	20	6.1	0.11	1.8	6.2	0.21	3.4
EDTA Blood							
-Healthy subject	20	2.6	0.08	3.1	2.7	0.13	4.8
-β-thalassemia carrier	20	6.4	0.16	2.5	6.47	0.29	4.5

matology Laboratory of the National Institute of Health by one medical scientist using one instrument.

1.1 **Within-run.** The study was performed using two different EDTA bloods and two different control solutions (Bio-Rad Laboratories, USA) with normal and increased values of Hb A_2. Each sample was performed in twenty replicates for each single run. Mean, SD and %CV were calculated and are summarized in Table 1. The within-run variability of %Hb A_2 was 1.8-3.1%.

1.2 **Between-run.** The study was performed using two different EDTA bloods and two different control solutions (Bio-Rad Laboratories, USA) with normal and increased values of Hb A_2 similar to those used for the within-run precision study. Each sample was analysed 4 times on 5 consecutive days. Mean, SD and %CV were calculated and are summarized in Table 1. The between-day variability of % Hb A_2 was 3.4-6.0%.

2. **Reproducibility.** Reproducibility study or interlaboratory comparison was performed using two different control solutions (Bio-Rad Laboratories, USA) with normal and increased values of Hb A_2. These quality control solutions were sent to 12 different laboratories using the Variant Hb testing system and 17 different laboratories using the HbGold analyzer. The variability of % Hb A_2 analyzed by Variant Hb testing system and HbGold analyzer were 4.0-7.1% and 6.8-8.8% respectively.

Accuracy: The comparison of methods experiment was performed to estimate the accuracy of %Hb A_2 quantitation. The Bio-Rad Variant hemoglobin testing system was used as the reference method. Correlation study revolving 90 EDTA blood samples of normal subjects and β- thalassemia carriers gave a linear regression equation of y = 0.97X-0.24. The correlation coefficient (r) was 0.98 (Fig 2).

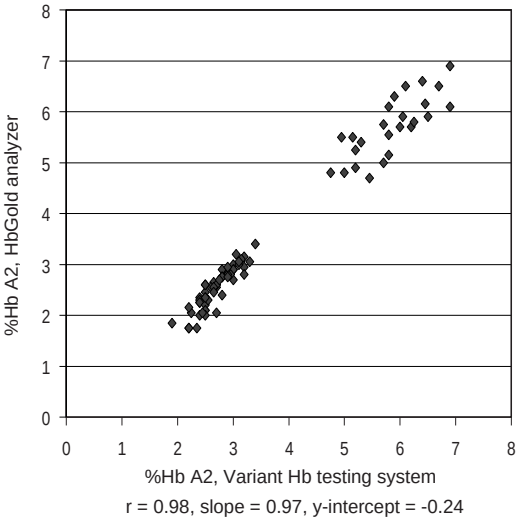


Fig 2–Comparison plot between %Hb A_2 of the HbGold analyzer and the Variant Hb testing system (N=90).

Thalassemia diagnosis by the Hb A_2 assay of the HbGold analyzer: The frequency distribution of %Hb A_2 was studied in 196 normal subjects and 127 β-thalassemia carriers (Fig 3). The concentration of Hb A_2 was 2.4±0.39% and 5.2±1.05% in normal subjects and β-thalas-

Table 2
Hb typing, %Hb A_2 and %Hb F of thalassemia phenotypes frequently observed in Thailand.

Phenotypes	N	Hb typing	Hb A_2/E, %	Hb F, %
Normal	196	A2A	2.4±0.39	0.6±0.84
β-thalassemia trait	127	A2A	5.2±1.05	2.2±2.17
Homozygous β-thalassemia	4	A2F(A)	2.3±1.39	69.3±25.79
Hb E trait	80	EA	28.2±7.47	1.9±1.44
Homozygous Hb E	18	E(F)	89.4±7.78	4.4±2.87
β-thalassemia/Hb E	30	EF(A)	47.4±15.57	34.3±14.68
Hb H disease	18	A2ABart'sH	1.4±0.89	< 0.5

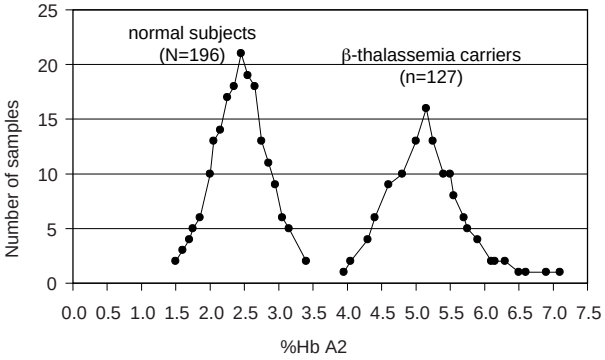


Fig 3–Frequency distribution of %Hb A_2 in normal subjects and β-thalassemia carriers.

semia carriers respectively. There was no overlap in the range of % Hb A_2 among these two subjects.

Table 2 and Fig 4 show the HbGold results obtained from blood samples of thalassemia phenotypes frequently observed in Thailand. In homozygous β-thalassemia the chromatogram illustrated the prominence of Hb F and the absence or decreased percentage of Hb A (Fig 4A). The chromatogram also showed the presence of Hb A, Hb E and Hb F in the patient with β^+-thalassemia / Hb E (Fig 4B). In Hb H disease, one or two peaks were eluted at the begining of the chromatogram. The two peaks represent Hb Bart's and Hb H, respectively (Fig 4C).

Presumptive identification of the commonly occuring hemoglobin variants, including Hb E, Hb C, Hb D and Hb S, could be made by using retention time windows, such as the E-window, C-window, D-window and S-window. Hb E, the most frequently occuring hemoglobin variant, eluted within the E-window (Fig 4D). The percentage of Hb E was 28.2+7.47% in a Hb E heterozygote and 89.4+7.78% in a Hb E homozygote. Many other abnormal hemoglobins were also seen: Hb Pyrgos, Hb J-Bangkok and Hb Constant Spring were eluted at 40-80, 160-190, and 290-340 seconds respectively.

DISCUSSION

The within-run, between-run and interlaboratory coefficients of variation (CV) of Hb A_2 quantitation were 1.8-3.1%, 3.4-6.0% and 6.8-8.8% respectively. The higher variability in the interlaboratory study might have been due to variations in ambient temperature: in high ambient temperatures, the retention time of Hb A_2 was lower. The HbGold is designed to operate in ambient temperatures from 15-30ºC; data from Drew Scientific indicated that at higher temperatures, the retention time was reduced and the Hb A_2 peak was lower and wider (Buck, 2001). Ambient temperature might vary over a day, on different days and in different laboratories: it is therefore necessary to run a calibration mixture before running a batch of samples. If the retention of Hb A_2 is shifted, it is necessary to change the temperature set point and optimize the instrument's performance. In addition, internal and external quality controls are also necessary to improve

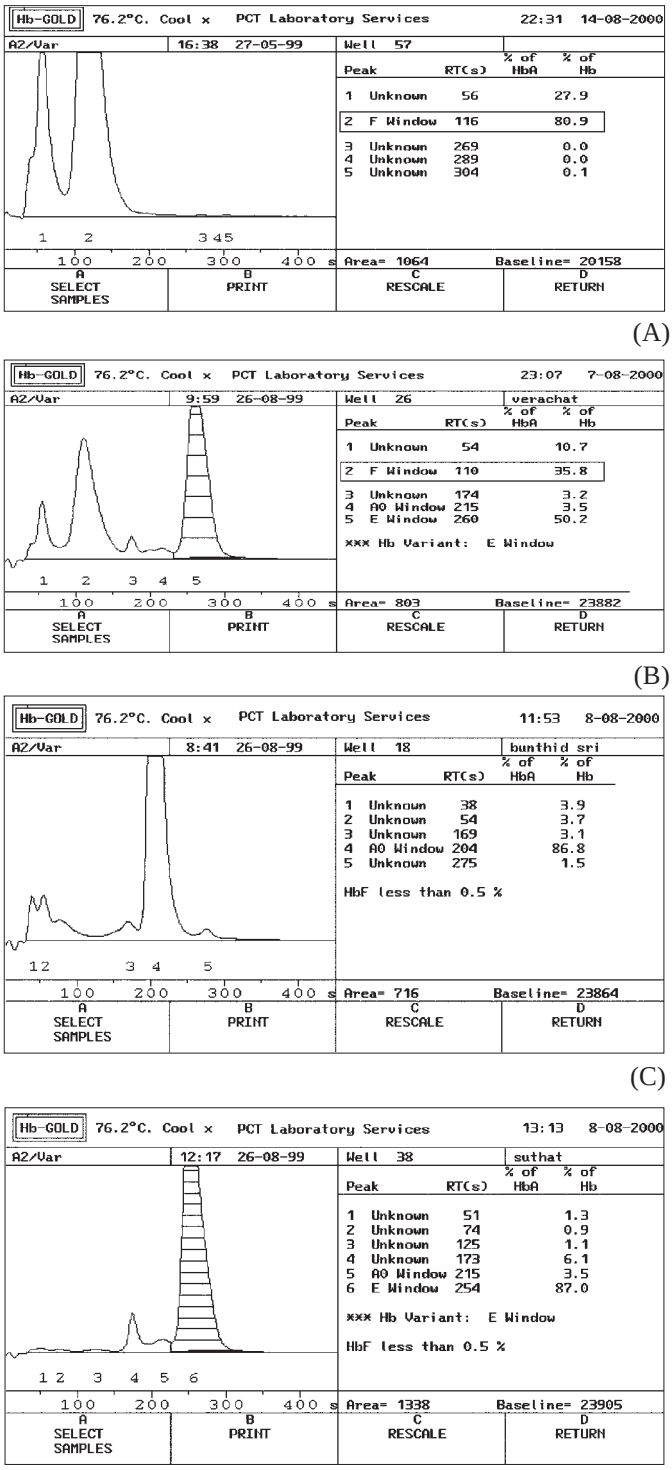


Fig 4–Chromatograms of homozygous β-thalassemia (A), β-thalassemia/HbE (B), Hb H disease (C) and homozygous Hb E (D).

laboratory performance.

The interlaboratory variability of %Hb A_2 analyzed by the HbGold analyzer was quite low. The College of American Pathologists hemoglobinopathy survey showed that the CV of %Hb A_2 by densitometric scanning of electrophoretic gel, microcolumn chromatography, and automated HPLC were 32.2%, 16.0-19.4%, and 6.3% respectively (CAP, 2000). Quantitation of %Hb A_2 by scanning densitometry is not recommended as the precision of this method is not sufficiently high to support a diagnosis of β-thalassemia trait (WHO, 1994; BCSH, 1998). In this study, the results of interlaboratory comparison showed that the low variability of measurement of %Hb A_2 by the HbGold analyzer was comparable with that of automated HPLC (Variant Hb testing system); in addition, data from the comparison of methods experiment indicated that the %Hb A_2 determined by the HbGold analyzer correlated well with that determined by the Variant Hb testing system (r=0.98). The frequency distribution of the %Hb A_2 was studied in 127 β-thalassemia carriers in comparison with 196 normal subjects. Fig 3 illustrates that there was no overlap in the range of %Hb A_2 results among normal subjects and β-thalassemia carriers. Elevation of the %Hb A_2, which is the most important feature in identifying β-thalassemia trait, was observed. A level of Hb A_2 above 3.5% is indicative of β-thalassemia trait (ICSH, 1978; Cao *et al*, 1998); however, it should be noted that β-thalassemia trait might be present with normal or borderline %Hb A_2 levels. These atypical carriers include heterozygotes for some mild β^+-thalassemia mutations such as CAP +1 (A-C) and IVS I nt6 (T-C) and the coexistence of δ-thalassemia or α-thalassemia (Galanello *et al*, 1994; BCSH, 1998; Cao *et al*, 1998). It is

therefore possible that the diagnosis of β-thalassemia trait may be missed in these atypical carriers; further investigations, such as hematological features, clinical manifestations and gene analysis, are necessary.

Application of the HbGold analyzer for the diagnosis of the thalassemias and hemoglobinopathies was studied (Table 2, Fig 1 and Fig 4): all the results were consistent with those of conventional methods and HPLC. In conclusion, the Hb A_2 assay of the HbGold analyzer could be used for the diagnosis of thalassemias and the presumptive identification of abnormal hemoglobins. If abnormal hemoglobin is identified, further identification by other methods, such as a battery of electrophoretic tests (citrate agar, cellulose acetate at alkaline pH, IEF and PAGE of globin chains) and electrospray mass spectrometry analysis, should be conducted. For the few cases in which the identity of abnormal hemoglobin remains uncertain, because at least one test result differs from those defining in our data bank files, further characterization might be done by protein structure analysis or by DNA analysis.

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