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 A₂ and Hb F are essential for the diagnosis of the thalassemias and hemoglobinopathies. Presented in this study is the validation of the the Hb A₂ assay of the HbGold analyzer, a fully automated liquid chromatography system for hemoglobin separation and quantitation. Variability of Hb A₂ 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 A₂ 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 A₂ assay of the HbGold analyzer could be used for the quantitation of Hb A₂ 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 (γ₄) and Hb H (β₄) 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 A₂ 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 A₂. 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
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 A₂ and Hb F. Conventional methods for quantitation of Hb A₂ 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 A₂, Hb F and other abnormal hemoglobins. Presented in this study is the validation of the Hb A₂ 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 A₂ 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 A₂ assay of the HbGold analyzer**

Hemoglobin separation and determination by the Hb A₂ 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 A2 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 A2 assay of the HbGold analyzer could be used to separate and quantitate Hb A2, 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 A2 and Hb A. The retention times of Hb A2 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 A2 was increased.

Performance characteristics of the Hb A2 assay of the HbGold analyzer

Precision: Two measures of precision including repeatability and reproducibility were made in order to assess the variability of Hb A2 quantitation.

1. Repeatability. Repeatability was performed under constant conditions in the He-
Validation of the HbGold Analyzer

Table 1
Within-run and between-run variability of %Hb A₂ by the HbA₂ assay of the HbGold analyzer.

<table>
<thead>
<tr>
<th>Sample types</th>
<th>N</th>
<th>Within-run</th>
<th></th>
<th>Between-run</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>%CV</td>
<td>Mean</td>
</tr>
<tr>
<td>Control I</td>
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<td>2.0</td>
<td>0.06</td>
<td>3.0</td>
<td>2.0</td>
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<tr>
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<td>0.11</td>
<td>1.8</td>
<td>6.2</td>
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<tr>
<td>EDTA Blood</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>- Healthy subject</td>
<td>20</td>
<td>2.6</td>
<td>0.08</td>
<td>3.1</td>
<td>2.7</td>
</tr>
<tr>
<td>- β-thalassemia carrier</td>
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<td>6.4</td>
<td>0.16</td>
<td>2.5</td>
<td>6.47</td>
</tr>
</tbody>
</table>

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 A₂. 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 A₂ 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 A₂ 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 A₂ 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 A₂. 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 A₂ 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 A₂ 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).

Thalassemia diagnosis by the Hb A₂ assay of the HbGold analyzer: The frequency distribution of %Hb A₂ was studied in 196 normal subjects and 127 β-thalassemia carriers (Fig 3). The concentration of Hb A₂ was 2.4±0.39% and 5.2±1.05% in normal subjects and β-thalassemi-
There was no overlap in the range of % Hb A\textsubscript{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\textsuperscript{+}-thalassemia / Hb E (Fig 4B). In Hb H disease, one or two peaks were eluted at the beginning of the chromatogram. The two peaks represent Hb Bart’s and Hb H, respectively (Fig 4C).

Presumptive identification of the commonly occurring 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 occurring 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 inter-laboratory coefficients of variation (CV) of Hb A\textsubscript{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\textsubscript{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\textsubscript{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\textsubscript{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
VALIDATION OF THE HbGOLD Analyzer

The interlaboratory variability of %Hb A₂, analyzed by the HbGOLD analyzer was quite low. The College of American Pathologists hemoglobinopathy survey showed that the CV of %Hb A₂ 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 A₂ 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 A₂ 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 A₂ 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 A₂ 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 A₂ results among normal subjects and β-thalassemia carriers. Elevation of the %Hb A₂, which is the most important feature in identifying β-thalassemia trait, was observed. A level of Hb A₂ 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 A₂ levels. These atypical carriers include heterozygotes for some mild β⁺-thalassemia mutations such as CAP+1 (A-C) and IVS 1 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 A₂ 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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REFERENCES


WHO. Human genetics and noncommunicable diseases. WHO Fact Sheet, 1999; 209.
