Evaluation of a Commercial Kit for Microchromatographic Quantitation of Hemoglobin A₂ in the Presence of Hemoglobin S

R. M. Bain and H. G. Brown

Commercial microcolumns introduced in 1976 by Helena Laboratories ("Hb A₂ Quik Column") and by Isolab, Inc. ("Quik-Sep") provide a rapid, simple, accurate method for quantitation of hemoglobin A₂ (Hb A₂). However, these kits cannot be used for the quantitation of Hb A₂ in the presence of slow-moving variants such as Hb S. Recently, Isolab, Inc., produced a new kit ("Quik-Sep Improved Hb A₂ Test") for quantitation of both Hb A₂ and Hb S. We compared results obtained with the new Isolab kit to results obtained with the original Tris/HCl method for quantitation of Hb A₂ and Hb S. Blood was drawn from persons with sickle cell trait (A/S), sickle cell anemia (S/S), sickle cell/beta thalassemia (S/beta⁺ thal) and sickle cell/beta⁰ thalassemia (S/beta⁰ thal) and percentages of Hb A₂ and Hb S were determined by each method. We found no significant differences in Hb A₂ percentages by the two methods, and the coefficients of variation were similar. Both methods showed only slight overlap of Hb A₂ values from subjects with some form of beta thalassemia and those with A/S or S/S. However, the Tris/HCl method consistently gave values for Hb S that were higher and closer to those expected, suggesting that the Isolab kit does not accurately quantify Hb S.

Additional Keyphrases: thalassemia · hemoglobinopathy · chromatography, column · “kit” methods

Quantitation of Hb A₂ in the beta thalassemias is of critical importance in establishing an exact diagnosis (1). The most common methods used for quantitating Hb A₂ include electrophoresis on cellulose acetate followed by densitometry (2-5); electrophoresis on cellulose acetate, followed by elution of the hemoglobin (Hb) bands (6); and microchromatography (7). In our hands, densitometry did not adequately distinguish between samples from normal persons and beta thalassemia carriers, but the other two methods gave acceptable results (8). The commercial microchromatographic kits introduced in 1976 (Helena Laboratories, Inc., Beaumont, TX 77704, and Isolab, Inc., Akron, OH 44321) provide a simple, rapid, accurate method for Hb A₂ quantitation (9). However, these commercial kits and the original microchromatographic method (7) are limited by inability to quantitate Hb A₂ in the presence of a slow-moving variant such as Hb S or Hb D. Two modifications have been suggested to remove this limitation. In one, a glycine/potassium cyanide buffer system is used (10). This method has been evaluated and found unsatisfactory (9). In the other modification a longer chromatographic bed is used (7); thus this procedure is more time-consuming.

In 1979, Isolab introduced a new microchromatographic kit ("Quik-Sep Improved Hb A₂ Test") based on a modification of the glycine method (11) for quantitating both Hb A₂ and Hb S. We have evaluated the ability of this new Isolab kit to measure Hb A₂ and Hb S in persons with sickle cell trait, sickle cell anemia, and sickle cell/beta thalassemia.

Materials and Methods

Blood samples were collected, with disodium ethylenediaminetetraacetate as anticoagulant. Donors represented four different genotypes. Eleven donors had sickle cell trait (A/S), with more Hb A than Hb S. One donor had a low mean cell volume, suggesting that she also had alpha thalassemia trait. Thirteen donors had more Hb S than Hb A and a microcytic, hypochromic blood picture with mean cell volumes ranging from 67 to 79 fl. These donors were classified as Hb S/beta⁺ thalassemia. Thirteen donors whose clinical and family histories were compatible were classified as homozygotes for Hb S (S/S). All had mean cell volumes greater than 85 fl, and none had detectable Hb A. Eleven persons with Hb S but no Hb A, and with microcytosis and mean cell volumes ranging from 63 to 80 fl, were classified as Hb S/beta⁰ thalassemia. Available family histories were consistent with this interpretation, but in some cases we could not rule out the possibility of homozygous Hb S with alpha thalassemia trait.

Stroma-free hemolysates were prepared as previously described (11) within seven days of drawing the blood. Using both methods, the same technologist then prepared columns, applied hemolysates, collected Hb fractions, measured absorbance at 415 nm with a Beckman Model 25 spectrophotometer, and calculated the proportions of Hb A₂ and Hb S. Two controls were run with each method. One had a low proportion of Hb A₂, and the other had an above-normal proportion of Hb A₂.

Quantitations of Hb A₂ and Hb S by microchromatography were performed with Tris/HCl as previously described (7). Columns were poured in 5-ml disposable pipets (Kimble) to a height of 16 cm. Hb A₂ and Hb S were eluted with Tris/KCN buffer adjusted to pH 8.35 and 8.2, respectively. The remaining hemoglobin was then eluted with buffer adjusted to pH 7.0. Hb A₂ and Hb S were measured with the Isolab columns according to the manufacturer's instructions. Columns were inverted and then allowed to settle before use. Clear hemolysates were then applied as directed. Hb A₂ was eluted with 4 ml of "Hb A₂ Elution Agent," Hb S was eluted with 4 ml of "Hb S Elution Agent," and the remaining hemoglobin...
Table 1. Quantitation of Hb A₂ by the Tris/HCl and the Isolab Methods

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>N</th>
<th>Method</th>
<th>Range</th>
<th>Hb A₂ %</th>
<th>SD</th>
<th>SEM</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Low control</td>
<td>10</td>
<td>Tris/HCl</td>
<td>1.6–2.3</td>
<td>1.98</td>
<td>0.257</td>
<td>0.081</td>
<td>13.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolab</td>
<td>1.1–1.9</td>
<td>1.71</td>
<td>0.233</td>
<td>0.074</td>
<td>13.6</td>
</tr>
<tr>
<td>High control</td>
<td>10</td>
<td>Tris/HCl</td>
<td>4.1–6.4</td>
<td>4.90</td>
<td>0.676</td>
<td>0.213</td>
<td>13.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolab</td>
<td>3.9–5.7</td>
<td>4.79</td>
<td>0.489</td>
<td>0.155</td>
<td>10.2</td>
</tr>
<tr>
<td>A/S</td>
<td>11</td>
<td>Tris/HCl</td>
<td>2.5–3.6</td>
<td>3.09</td>
<td>0.281</td>
<td>0.085</td>
<td>9.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolab</td>
<td>2.3–3.4</td>
<td>2.94</td>
<td>0.359</td>
<td>0.108</td>
<td>12.2</td>
</tr>
<tr>
<td>S/S</td>
<td>13</td>
<td>Tris/HCl</td>
<td>1.8–3.4</td>
<td>2.94</td>
<td>0.461</td>
<td>0.128</td>
<td>15.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolab</td>
<td>2.2–4.1</td>
<td>3.05</td>
<td>0.555</td>
<td>0.154</td>
<td>18.2</td>
</tr>
<tr>
<td>S/β¹²thal</td>
<td>13</td>
<td>Tris/HCl</td>
<td>3.9–5.8</td>
<td>4.96</td>
<td>0.659</td>
<td>0.183</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolab</td>
<td>3.9–6.0</td>
<td>4.96</td>
<td>0.646</td>
<td>0.179</td>
<td>13.0</td>
</tr>
<tr>
<td>S/β⁰thal</td>
<td>11</td>
<td>Tris/HCl</td>
<td>3.5–5.3</td>
<td>4.44</td>
<td>0.590</td>
<td>0.178</td>
<td>13.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolab</td>
<td>4.0–6.6</td>
<td>4.81</td>
<td>0.913</td>
<td>0.275</td>
<td>19.0</td>
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</table>

was eluted with 4 mL of “Other Hemoglobin Elution Agent.”

Programs from the Statistical Package for the Social Sciences (SPSS) (12) were used in analyzing data. Subprogram T-TEST was used to compare means and to perform paired t-tests. Correlation and regression coefficients were calculated by subprogram REGRESSION, and Spearman’s rank-order coefficient was calculated by subprogram NONPAR CORR.

Results

Table 1 shows the range, mean, standard deviation (SD), standard error of the mean (SEM), and coefficient of variation (CV) obtained for measurements of Hb A₂ by both methods. Mean Hb A₂ proportions in the controls and within the four phenotypic classes did not differ significantly between methods, and the CVs were similar in both methods.

Figure 1 shows the percentages of Hb A₂ obtained for the same sample by both methods. Regression of the proportion of Hb A₂ by the Isolab method on the proportion of Hb A₂ by the Tris/HCl method gave a slope of 1.01 (SD 0.071) and an intercept of 0.04 (SD 0.284), not significantly different from a slope of 1.0 and an intercept of 0.0 which would be expected if the two methods gave very close results. A paired t-test showed that the mean difference between Hb A₂ as measured by both methods was not significantly different from zero (mean 0.08; SD 0.49; t = 1.14, p > 0.05). There was a highly significant correlation between the proportions of Hb A₂ as measured by both methods (r = 0.90, p < 0.01). Because of the bimodality of the two distributions, the nonparametric statistic, Spearman’s rank-order coefficient, was also calculated and found to be highly significant (rₛ = 0.90, p < 0.01).

To determine whether both methods can distinguish thalassemic and non-thalassemic subjects, frequency histograms of the Hb A₂ values were constructed (Figures 2 and 3). The A/S subjects are pooled with the S/S subjects and the S/β⁺ thal subjects pooled with the S/β⁻ thal subjects for simplicity. There is a small amount of overlap between the distributions of Hb A₂ for thalassemic and non-thalassemic subjects as measured by both methods.

Table 2 summarizes our findings on measurement of Hb S by both methods. The two methods gave significantly different mean proportions of Hb S in all four phenotypic classes. The CVs were higher with the Isolab method.

Figure 4 shows the percentages of Hb S obtained for the same sample by both methods. The Tris/HCl method yielded a higher proportion of Hb S than did the Isolab method in all
cases. Regression of the proportion of Hb S by the Isolab method on the proportion of Hb S by the Tris/HCl method gave a slope of 0.70 (SD 0.113) and an intercept of -3.70 (SD 2.23). Thus, the intercept is not statistically different from 0, but the slope is significantly different from the expected value of 1.0. A paired t-test revealed that the mean difference between Hb S as measured by both methods differed significantly from zero (mean 23.2; SD 15.0; t = 10.7, p < 0.01). The correlation coefficient was 0.67, which is highly significant (p < 0.01).

**Discussion**

Our results indicate that the Tris/HCl method and the improved Isolab method perform equally well in quantitating Hb A₂ in the presence of Hb S. However, neither method completely distinguishes thalassemic and nonthalassemic samples. Those which fall in the “borderline” area (between 3.4% and 4.0% Hb A₂) need further evaluation before a diagnosis can be made. Hematologic, family, and clinical data are all useful in distinguishing among the alternatives.

One advantage of the Isolab column is its speed. Elution of the Hb A₂, Hb S, and remaining hemoglobin takes, at most, 2 h, whereas the Tris/HCl method may take up to 7 h. If Hb S need not be measured, the Isolab procedure can be completed in less than 90 min.

In their kit instructions, Isolab reports means of 2.9, 2.9, and 5.3% Hb A₂ for the A/S, S/S, and S/thal classes, respectively. Our results are similar; however, Isolab reports a wider range of Hb A₂ values than we found: 1.7–4.5% for the A/S class, 1.7–9.9% for the S/S class, and 3.1–8.5% for the S/thal class. These apparent differences may be due to variability among technologists and illustrate the need for each laboratory to determine its own normal and abnormal ranges. We have not evaluated variability among different lots of Quik-Sep columns, which might contribute to the wider ranges reported by Isolab.

The Isolab kit was not as effective in quantitating Hb S; it consistently gave low values and exhibited great variability. In our experience, the Tris/HCl method gives lower Hb S values than those obtained by electrophoresis on cellulose acetate followed by densitometry. This may be due to failure to elute the glycosylated forms of Hb S along with the Hb S. Thus, column chromatography may not be the most nearly accurate method for Hb S quantitation. However, the very low values obtained with the Isolab column suggest that the Hb S Elution Agent failed to elute all the Hb S.

The utility of the Isolab column thus depends on the laboratory’s individual needs. For determining genotype, we believe accurate quantitation of Hb A₂ to be more crucial than accurate quantitation of Hb S. Visual inspection of the cellulose acetate strip usually suffices to determine whether a sample has more Hb A than Hb S (as in sickle cell trait) or more Hb S than Hb A (as in S/beta⁺ thal). However, there are clinical situations in which the percentage of Hb S must be accurately measured. Persons with sickle cell anemia are often transfused until their proportion of Hb S is decreased to a predetermined value. Because the Isolab column appears

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**Table 2. Quantitation of Hb S by the Tris/HCl and the Isolab Methods**

<table>
<thead>
<tr>
<th>Phenotype</th>
<th>N</th>
<th>Method</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
<th>SEM</th>
<th>CV %</th>
</tr>
</thead>
<tbody>
<tr>
<td>A/S</td>
<td>11</td>
<td>Tris/HCl</td>
<td>25.5–48.0</td>
<td>36.7</td>
<td>5.4</td>
<td>1.64</td>
<td>14.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolab</td>
<td>13.3–28.4</td>
<td>22.6</td>
<td>4.9</td>
<td>1.48</td>
<td>21.7</td>
</tr>
<tr>
<td>S/S</td>
<td>13</td>
<td>Tris/HCl</td>
<td>63.3–91.3</td>
<td>78.2</td>
<td>7.9</td>
<td>2.19</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolab</td>
<td>18.6–75.7</td>
<td>47.7</td>
<td>19.9</td>
<td>5.51</td>
<td>41.6</td>
</tr>
<tr>
<td>S/β⁺ thal</td>
<td>13</td>
<td>Tris/HCl</td>
<td>54.9–70.2</td>
<td>61.6</td>
<td>4.4</td>
<td>1.23</td>
<td>7.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolab</td>
<td>6.5–55.5</td>
<td>38.4</td>
<td>14.2</td>
<td>3.93</td>
<td>40.0</td>
</tr>
<tr>
<td>S/β⁰ thal</td>
<td>11</td>
<td>Tris/HCl</td>
<td>69.3–86.5</td>
<td>80.8</td>
<td>4.8</td>
<td>1.44</td>
<td>5.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Isolab</td>
<td>32.5–75.2</td>
<td>57.2</td>
<td>14.2</td>
<td>4.30</td>
<td>24.9</td>
</tr>
</tbody>
</table>

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![Fig. 3. Frequency histogram of the Hb A₂ values as determined with the Isolab Quik-Sep Improved Hb A₂ Test](image)

For simplicity, the Hb A₂ values for the A/S and S/S classes have been pooled, and the Hb A₂ values for the S/beta⁺ thal and S/beta⁰ thal classes have been pooled. One S/S sample fell into the S/thal class by this method.

![Fig. 4. Percentage Hb S in each sample as measured by the Tris/HCl and Isolab Quik-Sep Improved Hb A₂ Test](image)

In all cases, the Tris/HCl method gave a higher proportion of Hb S than did the Isolab method. The mean difference in Hb S as measured by both methods was significantly different from zero (p < 0.01) by paired t-test. Regression analysis gave a slope of 0.7, significantly different from the expected slope of 1.0. This suggests that the Isolab method systematically underestimates the proportion of Hb S.
systematically to underestimate the proportion of Hb S, it
should not be used for this purpose.

We thank Dr. James Eckman for providing several samples and Ms.
Lorraine Bryan for assistance in obtaining these samples.

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Evidence That the Low-Affinity Folate-Binding Protein in Erythrocyte
Hemolysate Is Identical To Hemoglobin
Steen Ingemann Hansen, Jan Holm, and Jørn Lyngbye

Gel filtration studies on erythrocyte hemolysate demonstrated the
presence of a folate binding protein, apparently of the low-affinity type, that co-elutes
with hemoglobin. Further, the folate binder eluted with a low salt concentra-
tion after DEAE-Sepharose® CL-6B anion-exchange chromatography of erythrocyte hemolysate at pH 6.3. The chromatographic behavior of hemoglobin labeled with [3H] folate was so similar to that of the present binder as to suggest that the folate binder in erythrocytes is in fact
hemoglobin. The present study further substantiates this hypothesis. By the combined use of gel chromatography and anion-exchange chromatography we demonstrate that a low-affinity folate binder, probably identical to hemoglobin, is present in erythrocytes.

Materials and Methods
Labeled folate was supplied by the Radiochemical Centre, White Lion Road, Amersham, Buckinghamshire HP7 9LL, U.K. The following three types of radiochemical preparations were used, [2-14C]folic acid, potassium salt (cat. no. CFA. 333), with a specific activity of 55 Ci/mol and a radiochemical purity of 97-99%; [G-3H] folic acid, potassium salt (TRA. 34), with a spec. act. of 5 kCi/mol and a radiochemical purity of 95-97%; and [3', 7', 9-3H] folic acid, potassium salt (TRK. 212), with a spec. act. of 29 kCi/mol and a radiochemical purity 95-97%. Hemoglobin was obtained from Sigma Chemical Co., St. Louis, MO 63178.

Specimens of venous blood (EDTA stabilized), drawn from 10 healthy volunteers, were pooled. After centrifugation at 1500 x g for 15 min, the plasma and buffy coat were discarded, and the erythrocytes were washed five times in isotonic NaCl solution (9 g/L) to remove contaminating plasma. In the last wash solution, no albumin was detectable by “rocket” immuno-electrophoresis (13) with monospecific antibody against albumin (Dakopatt, Copenhagen). The washed erythrocytes were diluted with an equal volume of distilled water and repeatedly frozen and thawed five to 10 times. The resulting hemolysate (hemolysis confirmed by microscopic examination) was dialyzed overnight at 4 °C vs 0.17 mol/L Tris buffer,