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ICSH Recommendations for Assessing Automated High Performance Liquid Chromatography and Capillary Electrophoresis equipment for the Quantitation of HbA₂

Running Title
Assessing Automated HPLC and CE for HbA₂

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Introduction

\(\beta\)-thalassemia, an inherited recessive condition, is caused by one or more of over 200 mutations in the \(\beta\)-globin gene in man. It occurs in all populations, however it is much more common in some populations, ranging from 10% down to others with 1% carriers [1]. Many countries now screen couples before marriage or during pregnancy to assess whether or not they are at risk of having a child with \(\beta\)-thalassemia major (the severe homozygous or compound heterozygous state) since these conditions have significant lifelong medical morbidity and limited life expectancy. Although the precise characterization of the \(\beta\)-thalassemia mutation requires DNA analysis, the accurate measurement of HbA\(_2\) is the first level diagnostic parameter for the routine detection of most carriers of the common “High HbA\(_2\)” type of \(\beta\)-thalassemia. Most service laboratories and clinicians have to make a provisional diagnostic decision depending on the HbA\(_2\) level. As there might be only a small difference in the level of HbA\(_2\) between people who are carriers of High HbA\(_2\) \(\beta\)-thalassemia and those who are not carriers, the performance of the HbA\(_2\) analysis must be accurate and should be interpreted in...
association with the red cell indices. The red cell hematology should be that of a chronic microcytic state with moderate erythrocytosis relative to the hemoglobin concentration (unless folate deficient) and not responding to appropriate iron therapy. Although “HbA₂ levels above 3.5% are considered the standard cut off value, above which heterozygosity for 𝛽-thalassaemia is indicated” [2] a HbA₂ of 4% is usually considered a safe threshold to diagnose 𝛽-thalassemia trait with most carriers having levels of 3.8% or above; levels below 3.0% are usually considered safe to exclude cases of the common High HbA₂ 𝛽-thalassemia trait if normocytic and normochromic as most people who are not carriers have a HbA₂ less than 3.2%. However, even with greater accuracy and less imprecision the use of HbA₂ testing alone will not be able to exclude cases of borderline or “Normal HbA₂” 𝛽-thalassemia or combinations of 𝛽 and δ-thalassemia where a microcytic, hypochromic anemia with a normal HbA₂ might be mistaken for 𝛼-thalassemia. Because of this, individuals with suspicious red cell indices and normal ferritin and/or iron or zinc protoporphyrin levels deserve further evaluation. However, greater accuracy and less imprecision in the HbA₂ assay will both decrease needless testing from false positives and improve the detection of borderline cases. The level of the HbA₂ fraction may be measured as elevated in some non-𝛽-thalassemic circumstances or may be elevated due to technical artifacts [3]. For these reasons the HbA₂ accuracy is especially important in the critical area between 3.0 and 4.0% and the imprecision should be such that an SD of 0.05 (or CV, or RSD, of 2%) can be obtained (duplicates within 0.2% in the final numeric result). Unfortunately it is not possible to definitively diagnose or exclude a carrier state with a HbA₂ between 3.0 and 4.0% without further investigation. As stated previously [3] it is important to both detect and quantitate any HbA₂ variant that is present (due to either an 𝛼 or δ globin
chain mutation) and include it in the total HbA$_2$ reported. If this is not done, people who also carry β-thalassaemia, may be missed.

The International Committee for Standardization in Haematology (ICSCH) published recommended manual methods of measuring HbA$_2$ in 1978 [4] and discussed the various automated approaches in 2012 [3]. The ICSH Board have decided that since the various models of High Performance Liquid Chromatography (HPLC) and Capillary Electrophoresis (CE) equipment available from different manufacturers have been shown to give different values of HbA$_2$ in quality assessment schemes [5, 6], it would be useful to discuss how such equipment should be assessed by manufacturers of each system, as well as by service laboratories (especially before purchasing equipment), many of which do not have ready access to DNA analysis. Ideally, reliable HbA$_2$ standard materials should be available that are physically and biochemically identical to clinical samples analyzed by dedicated HPLC and CE devices and with the HbA$_2$ values obtained from electrophoretic separation on cellulose acetate membranes with elution and spectrophotometric measurement of native fractions [4] [7]. These standards could then be used by manufacturers in order to have a unique and reliable calibration with low, normal and elevated values. Automatic measurement of HbA$_2$ and hematological parameters should then be sufficiently accurate to provisionally diagnose, or exclude the carrier state of the common ‘High HbA$_2$’ β-thalassemia trait and should be at least as good as the manual but laborious methods used by experienced technologists. In complex situations such as in the presence of iron depletion, alpha thalassemia or ‘Normal HbA$_2$’ β-thalassemia, or δ-thalassemia which decrease the elevated HbA$_2$ level in β-thalassemia carriers, accurate diagnostic conclusions cannot be drawn without additional (DNA) analysis.
Aspects of analytical performance such as peak shape and especially tailing; integration mode, separation and baseline, technical accuracy, linearity, precision, carry-over and calibration of new columns and buffer, especially pH, batches need to be assessed by the technologist, to ensure that the instrument will be able to perform well and to fulfil the clinical need after installation and set up according to the instructions of the manufacturer [8]. It is also important that the total peak area is within that recommended by the manufacturer of the equipment otherwise the quantitation may be incorrect. In case of doubt, performance can be controlled by using samples of known value that should be certified by a National or an International body such as the World Health Organization (WHO) or donated in-house HbA$_2$ samples validated by a reference laboratory or by using remnant blood samples from previously measured fresh clinical samples. At the present time, (2015) the only international recognized reference material for HbA$_2$ is the WHO International Reference Reagent which has an assigned value of $5.3 \pm 0.066\%$ obtained by an international collaborative study using electrophoresis and elution, micro-column chromatography and HPLC. This is held by the UK National Institute for Biological Standards and Control (NIBSC) in Blanche Lane, South Mimms, Potters Bar, Herts, EN6 3QG; (www.nibsc.org). Controls made from samples obtained from members of staff can be useful for laboratories that do not have access to commercial controls or the National/International standards. Samples obtained by venesection of staff members will usually have stable HbA$_2$ levels as long as iron deficiency does not occur. For this reason blood donors and women of child bearing age should be avoided unless their blood count and iron status are confirmed as normal. Such material can serve as suitable controls since the material will be similar to the other samples analyzed. If this material can be validated by a reference laboratory, it can also be used as a secondary standard. A ‘Reference Laboratory’ should have
access to the WHO Reference material (or another internationally accredited reference material) and have experience of, and use, the cellulose acetate electrophoresis and elution technique recommended by ICSH [4] since automated HPLC or CE systems should not be used to assign values to samples for standardization purposes. The WHO material mentioned above has now been available for more than 25 years and is still stable, however the International Federation of Clinical Chemists (IFCC) is working on a new HbA\textsubscript{2} certified reference material as a primary standard[9]. The ICSH and IFCC are planning to form a joint working party on this subject and with a view to produce secondary standards for use by laboratories and manufacturers. When assessing automated commercial instruments for suitability to quantitate HbA\textsubscript{2} in order to diagnose or exclude the diagnosis of β-thalassemia trait it is important to assess the accuracy and imprecision in the diagnostic range and in particular in the critical range between 2.8 and 4.2% of the total hemoglobin. Within this critical range the imprecision should be ± 0.2% of the HbA\textsubscript{2} in the final numerical result which is the current precision that can be obtained by an experienced technologist using the recommended manual (although time consuming) methods [4].

**Assessment** [10] [11] [12] [13] As stated above, the technologist needs to assess separation and resolution (overlaps), peak shape (asymmetry), integration and baseline stability, carryover, accuracy, imprecision and linearity of the specific instrument used. The samples used for the following analyses must not contain any hemoglobin variant and the ‘blood’ diluent should contain no or very little HbA\textsubscript{2} (see below). Three replicates should be analyzed on at least three separate occasions on different days of the week.

**System suitability**

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It is essential that before performing the following validation steps the electro-mechanical components of the equipment (HPLC or CE) are operating in accordance with the manufacturer’s instructions (e.g. for HPLC flow rates are correct and constant, for CE voltages and current are stable; detectors have been set at the correct wavelengths). Eluents and buffers should be as supplied by the system manufacturer; or made in the laboratory using good laboratory practices when the pH should be checked with a fully and properly calibrated pH meter. Carryover may be minimised and resolution improved by regular cleaning of the sample lines as recommended by the instrument manufacturer. The following are basically testing the column and capillary performance.

**Peak shape, separation and baseline**  
Analyze a blood sample that does not contain a variant hemoglobin and check that the chromatogram (or electropherogram) shows complete separation of the HbA and HbA\(_2\) peaks with return to baseline between them and that the peaks are symmetrical.

**Imprecision**  
Measure one sample at least 3 times and then calculate the mean, range and SD. This is the intra-day variation. Repeat the measurement of the same sample on 3 occasions (inter-assay variation) and compare the mean, range and SD. Between assays the sample should be kept at 4°C.

**Carryover** [14] [15] [16]  
Carryover can lead to erroneous peak quantitation and even spurious peaks leading to diagnostic errors. It is therefore very important to know whether carryover occurs and if so what its clinical implications may be. When quantitating HbA\(_2\) it is especially important to know if there is any carryover from HbE or HbC since they elute with, or close to, HbA\(_2\) and HbA\(_2\) variants respectively on
HPLC and the peaks are at least ten to twenty times larger than the HbA2 peak so that even a small degree of carryover can cause a clinically significant increase in the HbA2.

If the HbA2 level is clinically important in a particular patient sample it is probably best to exclude any result that follows a sample containing HbE or HbC, or a variant hemoglobin that elutes in a similar position, and rerun the sample.

To assess the amount of HbA2 carryover that occurs requires two samples, one having a low or low normal HbA2 (aim for about 2%) labelled ‘L’ and another sample having a HbA2 considerable raised (aim for at least 5%) labelled ‘H’. These samples should be analyzed in the following way. Each sample should be analyzed three times in the following order: L1, L2, L3, H1, H2, H3 which shows the carryover from a normal to a thalassemia trait sample followed by H1, H2, H3, L1, L2, L3 to show the carryover from a thalassemia trait sample to a normal sample. The percentage carryover from the first group is calculated as follows:

\[ \frac{H1 - H3}{L3 - H2} \times 100\% \]
and the second group:

\[ \frac{L1 - L3}{H3 - L2} \times 100\% \]

A clinical laboratory should repeat the sequence of twelve assays five times. A manufacturer should plan to repeat the sequence at least twenty times. The mean carryover is then calculated for the two situations. A similar approach can be used to calculate the carryover from HbE or HbC to HbA2. The HbE or HbC sample should be at the highest level likely to be encountered in routine laboratory practice and will replace the ‘High’ raised HbA2 in the above example.
Carryover from the HbA2 should be less than a numeric 0.1% (equivalent to a carryover of 2% for a HbA2 of 5%). Since even this proportion of HbE would lead to a very large error in the HbA2 of a following sample, no carry over should be accepted from any variant eluting in the HbA2 region.

**Accuracy** Measure a reference material 3 times and also relate it to the linearity results.

**Linearity** Since for clinical reasons HbA2 is measured as a percentage of the total hemoglobin, the reconstituted WHO reference material (or any other reference material) should be stored in the manner indicated by the reference organisation and should be diluted in blood containing very little or ideally no HbA2 (see below) and not in water or buffer. Mix the reference material with the diluent in the proportions given in the table below and then analyse the mixture in duplicate on three occasions on different days of the week. In a similar way, undertake the cellulose acetate electrophoresis analyses. Plot the results against the theoretical value obtained by calculation (see table below). Ideally use a blood sample from a donor with homozygous delta thalassemia for all dilutions, but since this may be difficult to obtain, umbilical cord blood (preferably from a premature baby) can be used since this will contain very little HbA2 (usually less than 0.5%). The same pipette and settings should be used for all pipetting to minimize any calibration error. The hemoglobin concentration of donor blood sample should be similar (within 10%) to that of the WHO preparation (98 g/L) but the actual hemoglobin concentration of both should be measured using the same technique, since any difference in concentration will need to be taken into account to calculate the actual concentration of HbA2 in the mixture.
Integration Integration should employ a suitably fast data acquisition rate for HPLC see Meyer [17]. For CE a similar requirement applies to CE data systems but the relative areas must be calculated using ‘spacial areas’ (measured areas divided by the migration time).

Table 1 near here

Technique for linearity study

1. Open one ampoule of the WHO Reference Material (or other Reference Material) and reconstitute by adding 0.5mL distilled water to the vial. Mix well and when the lyophilised material has all dissolved it will produce 0.5ml of hemolysate at approximately 100g/L. If a different reference material is used reconstitute it using the appropriate recommended method.

2. Wash the red cells from 2ml ‘diluent’ blood three times with saline, centrifuge (1000g for 10 minutes) and remove the supernatant. Then haemolyse by adding an equal volume of water, mix and stand for 20 minutes, then freeze and thaw the red cells, then centrifuge (1000g for 10 minutes) to separate the red cell membranes, remove and keep the supernatant hemolysate.

3. Measure the Hb concentration of both the reconstituted WHO (or other) Reference Material and the haemolysed cord blood using the same equipment and reagents and record the results. Only use a very small volume of the reference material as most of
it will be needed for the linearity assessment.

4. Using the same pipette, prepare 6 dilutions of the WHO (or other) reference material as given in the Table. The same pipette should be used for all dilutions since this will reduce the effect of any inherent inaccuracies in the pipette calibration.

5. Analyse the HbA\textsubscript{2} in the undiluted WHO (or other) reference material & undiluted diluent blood and in the 6 dilutions from (4) above on the HPLC or CE equipment to be tested using the manufacturers directions for small (paediatric) samples. Also analyse each dilution by the ICSH recommended cellulose acetate electrophoresis and elution method [4].

6. Use the results from (3) and (5) above as described below to plot the linearity.

Linearity can be plotted as the dilution factor on the X-axis and the obtained HbA\textsubscript{2} percentage on the Y-axis. The expected HbA\textsubscript{2} percentage can also be plotted against the obtained HbA\textsubscript{2} percentage in order to assess the degree of correlation.

**Interpretation of results**

Peak shape, separation and baseline: When analyzing a sample that does not contain a variant hemoglobin, peaks should be Gaussian and symmetrical, with minimal and ideally no peak tailing [11]. The HbA\textsubscript{2} peak should be completely separated from the HbA (on CE) or HbAo (on HPLC) peaks, with the nadir between the two peaks on the baseline. The noise and drift of the baseline should be similar to that obtained when analyzing a buffer blank. Manufacturers should aim to supply columns that in their system have an asymmetry factor (As) between 0.9 and 1.1 and a resolution (Rs)
between adjacent peaks of greater than 1.75. These two factors are essential elements in assessing routine suitability of the separations obtained. These are described in detail in most HPLC texts such as Meyer [17].

When using manual techniques such as electrophoresis and elution that were recommended in the ICSH 1978 publication, complete separation of HbA₂ from HbA is possible, as is a relative standard deviation (similar to the CV) of 2.0%. Similar performance should be obtained by automated equipment in order to provide adequate clinical diagnoses.

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Conflict of Interest

The authors declare no conflict of interest.

Bibliography


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Table 1. Suggested mixing proportions for linearity testing.
Ideally, the diluent should be a blood sample from a person with homozygous delta-thalassemia, but if this is not available a sample of umbilical cord blood (preferably from a premature infant) can be used as this will usually contain less than 0.5% HbA₂.

The actual concentration of HbA₂ in the diluted samples is calculated (see below) from the ratio of WHO (or other) Reference material to diluent adjusted for the difference in hemoglobin concentrations. This calculation should not be used for the undiluted reference material or for the 100% diluent. If umbilical cord blood is used as the diluent there is likely to be a small amount of HbA₂ in the diluent and this is accounted for in the second part of the equation.

<table>
<thead>
<tr>
<th>WHO (or other) Reference Material volumes</th>
<th>Diluent * volumes</th>
<th>Approx HbA₂ concentration (%) §</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>0</td>
<td>5.3</td>
</tr>
<tr>
<td>4</td>
<td>1</td>
<td>4.2</td>
</tr>
<tr>
<td>5</td>
<td>2</td>
<td>3.8</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>3.5</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>3.3</td>
</tr>
<tr>
<td>4</td>
<td>3</td>
<td>3.0</td>
</tr>
<tr>
<td>2</td>
<td>3</td>
<td>2.1</td>
</tr>
<tr>
<td>0</td>
<td>5</td>
<td>0</td>
</tr>
</tbody>
</table>

* Ideally, the diluent should be a blood sample from a person with homozygous delta-thalassemia, but if this is not available a sample of umbilical cord blood (preferably from a premature infant) can be used as this will usually contain less than 0.5% HbA₂.

§ The actual concentration of HbA₂ in the diluted samples is calculated (see below) from the ratio of WHO (or other) Reference material to diluent adjusted for the difference in hemoglobin concentrations. This calculation should not be used for the undiluted reference material or for the 100% diluent. If umbilical cord blood is used as the diluent there is likely to be a small amount of HbA₂ in the diluent and this is accounted for in the second part of the equation.
HbA$_2$ percentage in the mixture = $[A \times B/(B+C) \times D/E] + [F \times C/(B+C) \times E/D]$

Where:  
A = HbA$_2$ percentage in the WHO (or other) reference material  
B = Volume of ‘Reference material’  
C = Volume of ‘Diluent’  
D = Hb concentration of ‘Reference material’  
E = Hb concentration of ‘Diluent’  
F = HbA$_2$ percentage in the ‘Diluent’