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ICSH recommendations for the measurement of Haemoglobin A_2

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SUMMARY

Although DNA analysis is needed for characterization of the mutations that cause β-thalassaemia, measurement of the Hb A_2 is essential for the routine identification of people who are carriers of β-thalassaemia. The methods of quantitating Hb A_2 are described together with pitfalls in undertaking these laboratory tests with particular emphasis on automated high-performance liquid chromatography and capillary electrophoresis.

INTRODUCTION

It is now 30 years since the first recommendations were published concerning the quantification of Haemoglobin A_2, and during this time, there have been several new analytical developments in the field; therefore, the International Council for the Standardisation of Haematology (ICSH) Board consider that the original recommendations should be revised. Haemoglobin A_2 (Hb A_2) is a haemoglobin tetramer composed of two α and two δ-globin chains ($\alpha_2\delta_2$). It is a normal haemoglobin that is not present in the fetus but gradually develops after birth increasing in concentration as δ-chain production occurs. The concentration of Hb A_2 (the minor adult haemoglobin) is raised in most carriers of β-thalassaemia, and the correct quantification of Hb A_2 is essential for the routine diagnosis of β-thalassaemia trait. As β-thalassaemia major has major clinical implications for affected individuals and their families, it is important to detect the presence of β-thalassaemia trait in prospective parents so as to identify couples at risk of having a child with β-thalassaemia major. In countries where β-thalassaemia is a significant national health problem, such as Cyprus, premarital screening tests have been recommended since the 1970s. In some countries such as Iran, UAE and Bahrain, similar screening has only recently been introduced. In some countries (such as England), such a test has to be offered to all women in the early stages of pregnancy, but they are free to decline this testing if they so wish. In many countries in SE Asia where thalassaemia is prevalent, upon booking at the antenatal clinic, all pregnant women with mean cell volume (MCV) <80 fl or mean cell haemoglobin (MCH) <27 pg, but with normal iron status, will have the Hb A_2 quantification undertaken to help identify their thalassaemia status. In some other countries, it is up to the individual and/or doctor to decide if, and when, to undertake such tests.

Over the last 30 years, great strides have been made in the screening programmes for the haemoglobinopathies. Despite a wealth of accumulated data, several problems still remain in carrier identification. The most common issue is the presence of mild or moderate microcytosis with normal Hb A_2 and Hb F that may be due to iron deficiency, α-thalassaemia trait, 'normal-Hb A_2' β-thalassaemia, δβ-thalassaemia deletions, or a combination of these with common, or uncommon, δ-thalassaemia or δ-chain variants or more complex conditions that may interfere with Hb A_2 measurement (Bouva *et al.*, 2006; Phylipsen *et al.*, 2011). It is well known that iron deficiency anaemia results in a wide range of red cell anomalies such as reduction in MCV, MCH, RBC and Hb, depending on the severity at the time of analysis, whereas in carriers of thalassaemia, MCH, MCV and the Hb are reduced but the red cell count is relatively high compared with the haemoglobin level. Iron deficiency anaemia may be confused with α-thalassaemia or β-thalassaemia, and therefore, it is helpful to undertake iron status studies (such as serum ferritin, serum iron with transferrin saturation and/or zinc protoporphyrin). In some cases, 'high Hb A_2 β-thalassaemia' may occur together with iron deficiency anaemia, and in most of these cases, the level of Hb A_2 will remain elevated but in some people with 'borderline Hb A_2 beta-thalassaemia', a significant iron depletion may reduce the slightly elevated Hb A_2 level into the normal range. In such situations, it would be advisable to treat the patient with iron to correct anaemia before determining Hb A_2 levels. This is particularly important in regions where both iron deficiency anaemia and α-thalassaemia coexist in epidemic proportions such as in the United Arab Emirates where the frequency of the α-thalassaemia gene among the national population is estimated at around 50% through DNA studies (Baysal, 2001).

In the diagnosis of β-thalassaemia trait, it is the proportion of Hb A_2 relative to any other haemoglobin present, not the absolute amount of Hb A_2, that is clinically important, and if a Hb A_2 variant is present (because of an α or δ-globin chain variant), it is the total Hb A_2 that is diagnostic. It is therefore analytically important to measure all the Hb A_2 and any other haemoglobin present (usually Hb A and Hb F and not infrequently a Hb A_2 variant haemoglobin) to calculate the relative proportion of Hb A_2. It is equally important not only to select a method that can detect and quantify any Hb A_2 variant but also for the analyst to be aware of the need to incorporate them in any analytical procedure. Some variant haemoglobins will interfere with the quantification of Hb A_2; for instance, Hb C and E migrate with Hb A_2 on cellulose acetate electrophoresis at alkaline pH whereas Hb Lepore, Hb E and sometimes

Hb D may co-elute with Hb A_2 on many dedicated high-performance liquid chromatography (HPLC) systems. Some commercial cation-exchange HPLC systems optimized for the quantification of Hb A_{Ic} have compressed the later part of the chromatogram to reduce the cycle time, and these are likely to miss many Hb A_2 variants.

Kunkel recorded the presence of a minor haemoglobin band on electrophoresis of adult blood (Kunkel & Wallenius, 1955) and subsequently showed that it was raised in the parents of people with thalassaemia (Kunkel *et al.*, 1957). In people who have the common types of β-thalassaemia trait, the Hb A_2 concentration is usually between 4.0% and 6.0% and is rarely outside the range of 3.5–7.0% of the total haemoglobin. In people who do not have this condition, the Hb A_2 concentration is usually between 2.2% and 3.3% and is rarely outside the range of 2.0–3.5% unless severe iron deficiency anaemia is present (Figure 1). This means that it is essential that the measurement of Hb A_2 is both precise and accurate and as stated earlier, if a Hb A_2 variant is present, it is the total Hb A_2 that has diagnostic significance. Manual assays were developed in the 1960s and 1970s, which can give clinically useful results of Hb A_2 levels, and these were reviewed by ICSH and published as recommended methods in 1978 (International Committee for Standardization in Haematology, 1978).

It is becoming apparent that many β-thalassaemia mutations associated with 'borderline' Hb A_2 levels that are sometimes referred to as 'mild thalassaemia mutations' can in fact be the cause of severe transfusion-dependent conditions in the homozygous state or in combination with other β-thalassaemia defects, with all the potential problems associated with β-thalassaemia major (Old *et al.*, 2008). Some β-thalassaemia carriers have normal or borderline Hb A_2 levels but MCV and MCH within the typical carrier range. Such carriers may include heterozygotes for some 'mild mutations', such as IVS-I-6 (T→C), double heterozygotes for δ- and β-thalassaemia (*in cis or in trans*), carriers of the Corfu δβ-thalassaemia and εγδβ-thalassaemia heterozygotes. The latter involve very large deletions of the β-globin gene cluster, some sparing the β-globin gene (English, Hispanic) and others deleting it (Dutch, Scottish–Irish, Mexican, Irish, Canadian, Croatian). Most of these mutant alleles are summarized in Table 1.

Family studies and DNA analysis are strongly recommended to differentiate these atypical β-thalassaemia carriers from α-thalassaemia heterozygotes. Atypical carriers with 'very mild' or 'silent' β-thalassaemia mutations (FBC and Hb A_2 levels are borderline/normal) may also include carriers of the triple α-globin gene rearrangement (Giordano, Bakker-Verwij & Harteveld, 2009). Identification of a 'silent' β-thalassaemia carrier is often retrospective in parent(s) of patients with moderate/mild β-thalassaemia intermedia. Generally, if a silent β-thalassaemia mutation is suspected, this is best ascertained through DNA analysis such as β gene sequencing. Because of their 'silent' phenotype, these carriers may escape identification in general population screening programmes. In

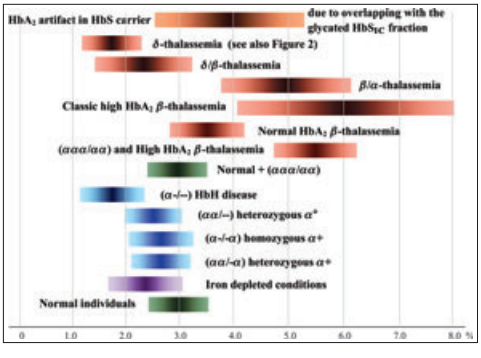

Figure 1. Ranges of Hb A_2 values measured in 766 individuals from different cohorts including 32 cases of α triplications.

Table 1. Some β-thalassaemia mutations known to be associated with a borderline Hb A_2 levels (Old *et al.*, 2008)

CAP+1 (A→C)
−101 (C→T)
−92 (C→T)
PolyA (−AT)
PolyA (A→G)
PolyA (−AA)
PolyA (T→C)
IVS-I-6 (T→C)
IVS-II-844 (C→G)
+1480 (C→G)
+33 (C→G)

specialized laboratories, every suspected case is analysed at the molecular level by DNA techniques. It must be emphasized that in multi-ethnic populations like those emerging in countries with high levels of immigration, the spectrum of mutations can be much larger with the likelihood of gene admixture than in countries where thalassaemia is endemic.

In all these cases, it is extremely important that the Hb A_2 assay is both accurate and precise as otherwise these cases may be missed until the affected people produce a child with transfusion-dependent thalassaemia. Complicated thalassaemia genotypes can result in borderline Hb A_2 levels and also cause diagnostic problems that may require detailed DNA analysis to elucidate as mentioned earlier (Paglietti *et al.*, 1985; Galanello *et al.*, 1994; Giambona *et al.*, 2008).

PRE-ANALYTICAL CONSIDERATIONS

In 1993, it was documented that the Hb A_2 concentration may be raised in HIV during treatment (Galacteros *et al.*, 1993; Routy *et al.*, 1993; Pornprasert *et al.*, 2009). Other causes of a raised level are thought to be very unusual (Weatherall & Clegg, 2001) but are listed in Table 2. Severe iron deficiency anaemia can reduce the Hb A_2 level, (Wasi, Disthasongchan & Na-Nakorn, 1968; Kattamis *et al.*, 1972; Cartei & Dini, 1975; Cartei *et al.*, 1976; Steinberg, 1993) and in some reports, this has been shown to interfere with the diagnosis of beta-thalassaemia trait while others have stated that the effect is not severe enough to affect such diagnosis (Galanello *et al.*, 1990; Steinberg, 1993; Madan *et al.*, 1998). Other conditions have been documented as reducing the Hb A_2 level and are listed in Table 3.

Table 2. Main pre-analytical subject-related factors that may ***increase*** Hb A_2 levels

Thalassaemic syndromes	Heterozygous β-thalassaemia
Other haemoglobinopathies	Artefact in the presence of Hb S
	Some haemoglobin variants with thalassaemic phenotype
Acquired conditions	Hypertrophic osteoarthropathy
	Megaloblastic anaemia
	Pseudoxanthoma elasticum
	Hyperthyroidism
Treatment-related situations	Antiretroviral therapy in patients with HIV

Table 3. Main pre-analytical subject-related factors that may normalize or ***decrease*** Hb A_2 levels

Thalassaemic syndromes	'Silent' β-thalassaemia alleles
	Interaction between δ- and β-thalassaemia (δ+β-thalassaemia)
	δβ-thalassaemia
	Hb H disease and other α-thalassaemias
Other haemoglobinopathies	α-chain variants
	δ-chain variants
Acquired conditions	Erythroleukaemia
	Severe iron-deficiency anaemia
	Sideroblastic anaemia

SAMPLE COLLECTION AND STORAGE

Any anticoagulant can be used although it is common to use K_2EDTA, which is the anticoagulant used for the analysis of blood counts. Ideally the analysis should be undertaken as soon as possible after collection but storage of a sealed whole blood sample at 4 °C for 2–3 weeks is acceptable for Hb A_2 as there will be minimal oxidation at 4 °C during that time (Tietz, 1990). Where there are high ambient temperatures, such as in tropical areas, suitable means of transport must be used to prevent deterioration of the haemoglobin because considerable denaturation occurs even after 1 h at 50 °C. Sample stability after freezing at −20 or at −80 °C is not well documented although some manufacturers claim an overall stability of 1 month at −20 °C and 3 months at −80 °C. When freezing samples, it is essential that the blood samples are frozen as quickly as possible because slow freezing of proteins promotes denaturation; one satisfactory way is to freeze drops of whole blood or haemolysate aliquots in liquid nitrogen. Thawing and refreezing of blood samples are discouraged. A partially coagulated blood sample should not be accepted for Hb A_2 quantification by automated equipment as it may contaminate or block the tubing or column.

EXPRESSION OF RESULTS

To be clinically useful, the results of the Hb A_2 quantification have to be expressed as a percentage of the total haemoglobin (which will usually include the integration of the Hb A, Hb F, Hb A_2 and any glycated or aged adducts). However, in case of elevated baselines, one must be careful not to integrate disproportional areas (see more under peak quantification).

REFERENCE INTERVALS

The relative amount of Hb A_2 in adult healthy subjects, who are not carriers of β-thalassaemia, is usually between 2.0% and 3.3% (Weatherall & Clegg, 2001), and some slightly different numbers can be found in other contexts, depending on the measurement procedures and on differences in local populations. As an example, reference intervals were between 1.9% and 3.1% in a study on a Sardinian population (Galanello *et al.*, 1994). Other reference laboratories exclude from their normal cohort iron depleted and alpha-thalassaemia individuals and consider the variability among different instruments, use normal reference intervals between 2.5% and 3.5% (Van Delft *et al.*, 2009) see Figure 1.

NEWBORN β-THALASSAEMIA CARRIERS

Because of the reduced expression of the δ-globin gene with respect to the β gene, the Hb A_2 levels at birth are very much lower than in the adult life and are not measurable by standard methods, but normalize by 12 months of age. In β-thalassaemia carriers, Hb A_2 may increase with respect to normal subjects already by 3 months after birth (Mosca *et al.*, 2009) but does not reach levels found in adults until at least 12 months (Weatherall & Clegg, 2001). Alternatively, the level of Hb A has been used at birth to predict with an acceptable probability, the carrier status of β-thalassaemia in the newborn and confirmed by DNA analysis (Mantikou *et al.*, 2009).

PITFALLS

As mentioned in 'analytical considerations' and summarized in Tables 2 and 3, a number of conditions may either elevate or reduce the Hb A_2. One of the commonest causes of a falsely raised Hb A_2 is the presence of 'aged' Hb S or Hb S_{Id}, which frequently elutes in the same position as Hb A_2. The commonest cause of a reduced Hb A_2 is the presence of an α-chain variant that will produce a Hb A_2 variant, which may not be recognized or quantitated. Another cause of a Hb A_2 variant is a δ-chain variant, which again may not be recognized or quantitated. Finally, the presence of δ-thalassaemia will reduce the Hb A_2 even in the presence of δβ-thalassaemia. The implications in interpreting the results in these cases are summarized in Figure 2.

PEAK QUANTIFICATION

With automated systems such as HPLC, capillary zone electrophoresis (CZE) and capillary isoelectric focussing (cIEF), the accuracy of peak quantification is dependent on both peak resolution and the method of peak integration. Poor peak quantification will result in poor accuracy but may still result in good precision that may mislead analysts into believing that the accuracy is also good. It is important that the sample loading is within the manufacturer's recommended range as not only must the column not be overloaded but it is essential that the optical absorbance must be kept within the linear range because, if too much or too little sample is applied, any peaks may be under- or overestimated leading to the wrong relative estimation of small peaks such as the Hb A_2. As mentioned earlier, it is equally important that the baseline used for integration is correct as only those parts of the peak that are above the baseline will be quantitated, and this may lead to parts of a peak being excluded from the integration if the baseline is incorrectly assigned. As a general rule, with the equipment used for quantitating Hb A_2, the baseline should be straight and close to the horizontal. Ideally, the baseline should be obtained by running a blank sample twice (so that there is no haemoglobin present) at the beginning of each batch and recording the chromatogram produced with the second blank (to avoid any carry over), and this should be used as the baseline for that batch of samples. If a haemoglobin peak (such as Hb H) elutes with the void volume and if it occurs with some systems, the integration excludes the 'void peak', then in the presence of Hb H, the total amount of haemoglobin will be underestimated leading to an

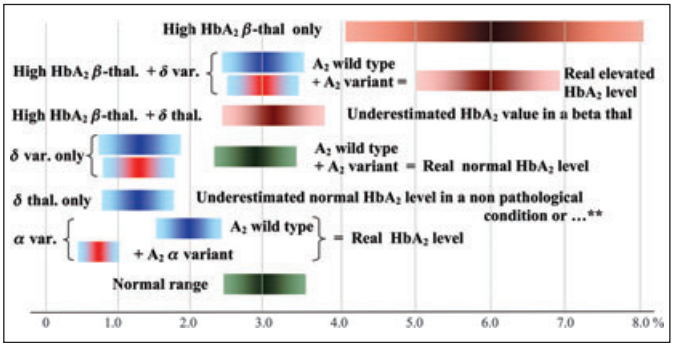

Figure 2. α variants and δ-globin gene defects (δ variants and δ-thalassaemias) and their implications interpreting the Hb A_2 levels in β-thalassaemia diagnosis.

overestimation of the Hb A_2 peak. Accurate quantification of a peak also requires that that peak is completely separated from neighbouring peaks, and this is especially important for small peaks such as Hb A_2. For all these reasons, it is extremely important that the chromatogram, or electropherogram, is inspected carefully before the results are authorized. As Hb A_2 is reported as a ratio of Hb A_2 to the total haemoglobin, as long as there is an appropriate baseline associated with good peak integration, area calibrators should not be necessary as they are a poor substitute for inadequate chromatography or peak quantification. However, standards and controls remain essential to verify that the equipment is working satisfactorily. Although for HPLC and cIEF it is the peak areas that are related to the amount of haemoglobin present, it should be noted that in capillary zone electrophoresis, the peak areas should not be used for quantification owing to the different migration velocities through the detector, instead the 'spatial areas' (integrated area divided by the migration time) should be used (Huang, Coleman & Zare, 1989; Hempe & Craver, 1999).

PRECISION, ACCURACY AND DIAGNOSIS

As stated earlier, both precision and accuracy are important because a raised level of Hb A_2 is a diagnostic marker for beta-thalassaemia trait. However, measurement of the Hb A_2 level alone cannot absolutely confirm or exclude the carrier state, because there may be very little numerical difference between the Hb A_2 level in people with and without β-thalassaemia trait. Nevertheless, precision is important, and the level obtainable with manual techniques is ±0.1% in the final answer, which is equivalent to an SD of 0.05% (leading to a CV of 2% in the normal range and 1% in the thalassaemia range). A similar precision level should be obtainable in the modern automated HPLC and capillary electrophoretic equipment. Accuracy is harder to establish and monitor but comparison with the WHO Standard Reference Material (see Conclusions below) and/or comparison with National or Regional quality assessment schemes can be helpful. Investigations are in progress to use mass spectrometry to assess candidate standards. Concerning the relative total error, an acceptable limit of 7.0% has been recently proposed (Mosca *et al.*, 2009), to correctly classify a subject with a true Hb A_2 value of 3.6% besides other variables, such as MCV and MCH. Indeed, in this case, the measurement error should not exceed 0.25% (relative total error 7.0%) to avoid the possibility of misclassifying an individual on the basis of a Hb A_2 measurement as a putative β-thalassaemia carrier (Hb A_2 >3.8%) or as a non-β-thalassaemia subject (Hb A_2 <3.3%).

ANALYTICAL TECHNIQUES

As stated earlier, the analytical principle consists of separation and quantification of Hb A_2 (and any Hb A_2 variant) in relation to all other haemoglobins present. The haemoglobin fractions can be separated by electrophoresis and elution, by anion-exchange microcolumn chromatography, by cation-exchange HPLC,

by CZE or by cIEF. Quantification is usually undertaken by spectrophotometric detection of the separated bands or peaks at 415 nm, which is the absorption maximum for oxyhaemoglobin. It is important to ensure that bilirubin does not interfere with this analysis because it also absorbs at this wavelength. Packed, washed red cells are used to prepare the haemolysate for electrophoresis and for microcolumn chromatography, the two methods recommended by the ICSH in 1978 which will remove bilirubin. When HPLC is used, bilirubin elutes very early with the void peak, and therefore, some instruments do not include this area in the integration programme. Hb Barts and Hb H also elute with the void peak. With capillary electrophoresis, the order of migration is similar to alkaline electrophoresis. In some new CZE instruments, the resolution has been improved so that Hb A_2 is resolved from Hb E. This is also true for some HPLC systems provided by some manufacturers. Some analytical factors that affect Hb A_2 quantification are given in Table 4, and some of the haemoglobin variants that elute with, or close to Hb A_2, are given in Tables 5 and 6. The methodological details for electrophoresis and for microcolumn chromatography are given in the previous ICSH recommendations (International Committee for Standardization in Haematology, 1978) both of which can give reliable results with experienced analysts.

With the huge increase in throughput of Hb A_2 measurements in hospital laboratories because of both

Table 4. Main analytical factors affecting the Hb A_2 quantification

The presence of an α or δ variant haemoglobin
A haemoglobin variant eluting, or migrating, with Hb A_2
Too large, or too small a sample application
Incorrect buffer or column temperature*
Inadequate separation of the Hb A_2 from Hb A (or any other haemoglobin)
Inappropriate baseline†
Inappropriate integration†

CZE, capillary isoelectric focussing; cIEF, capillary isoelectric focussing; HPLC, high-performance liquid chromatography.
*HPLC, CZE and cIEF only.
†HPLC and manual microcolumn chromatography.

Table 5. Some variant haemoglobins that elute with, or close to, Hb A_2 on HPLC

Hb Abington*
Hb Abruzzo
Hb Akron
Hb Boras
Hb Bethesda*
Hb Chandigarth
Hb Deer Lodge
Hb D Iran*
Hb Denver*
Hb D-Ouled Rabah
Hb E
Hb Ethiopia*
Hb Fort Worth
Hb G Copenhagen
Hb G Coushatta*
Hb G Ferrara
Hb G Galveston
Hb G Honolulu*
Hb G Taipei
Hb Hoshida
Hb Hamadan
Hb Jeddah
Hb Kenya
Hb Korle Bu*
Hb Lepore Baltimore
Hb Lepore Boston
Hb Lepore Hollandia
Hb Loves Park*
Hb M Saskatoon
Hb Muravera
Hb Nebraska
Hb Ocho Rios
Hb Osu Christiansborg*
Hb Paddington
Hb Rocky Mountain
Hb San Bruno*
Hb Santa Juana*
Hb SId (the aged adduct of Hb S due to glutathione)
Hb Spanish Town
Hb Toulon
Hb Tubingen
Hb Zurich

HPLC, high-performance liquid chromatography.
*These haemoglobin variants are sometimes labelled Hb A_2 and sometimes unknown depending on the instrument and slight variations in retention times. Even when the variant does not precisely co-elute with Hb A_2, it may still interfere with the Hb A_2 measurement and so a different method of measuring the Hb A_2 should be used in this situation.

Table 6. Some variant haemoglobins that migrate with, or close to, Hb A_2 on capillary zone electrophoresis

Hb Chad
Hb E-Saskatoon
Hb O-Arab
Hb C Harlem*

*Insufficient separation for accurate quantification.

the mandatory and the voluntary screening programmes, automated HPLC or capillary zone electrophoresis has become the main tool to quantify Hb A_2 in most laboratories in Europe and North America and are being increasingly used in Asia and Africa, and capillary isoelectric focussing is also being introduced. However, dedicated instruments are available from different companies and these approach the analytical issues in different ways (Van Delft *et al.*, 2009). Some of these automated HPLC instruments are primarily designed to quantify Hb A_{Ic} but are also used to quantify Hb A_2. When these instruments are used to measure Hb A_2, it is essential that the columns and buffers are optimized for the Hb A_2 measurement. Automated instruments are also used as a first-line screen for variant haemoglobins such as Hb S, C, D-Punjab, O-Arab and E, and if these haemoglobins are present, they may interfere with the measurement of the Hb A_2. However, this fact should not represent a diagnostic problem because the product of two beta genes (Hb A and Hb S, C, E, O or another beta chain variant) confirms the activity of both beta genes and thus the absence of β-thalassaemia and makes the separation and measurement of the Hb A_2 fraction for β-thalassaemia diagnostics obsolete in these cases (Van Delft *et al.*, 2009). Quality assessment programmes have shown considerable variation in the Hb A_2 levels reported by HPLC analysis from aliquots of the same blood sample (Batterbee *et al.*, 2010). Interlaboratory variation among users of three HPLC systems produced by the same manufacturer has been reported between 6.0% and 9.6% (expressed as CV) (Paleari *et al.*, 2007). To minimize inter- and intralaboratory variation, it is important to use the internal controls provided by the manufacturers correctly. Automated capillary electrophoresis is becoming available and is claimed to be equally satisfactory for measuring Hb A_2 and also for screening of variant haemoglobins such as Hb S, C, D-Punjab, E and O-Arab. However, in spite of these analytical changes, there have not been any recommendations as to how such equipment should be assessed and selected by service, referral or research laboratories. In 1988, the WHO agreed on the value of the first International Reference Material for Hb A_2 (World Health Organization Expert Committee on Biological Standardization, 1994). This is a stabilized freeze-dried preparation (89/666) prepared from blood obtained from the mother of a patient with beta-thalassaemia major and is held at National Institute of Biological Standards and Controls (NIBSC) South Mimms, Herts, UK. The value assigned to this preparation was obtained by an international collaborative study using the methods recommended by ICSH in 1978 and also by the HPLC equipment that was commercially available at that time. As it is now 20 years since this international reference material was prepared and validated, another preparation is being developed (Paleari *et al.*, 2010) that will be assayed by mass spectrometry and by the original ICSH recommended methods and by HPLC and CZE as well.

HISTORICAL PERSPECTIVE OF ELECTROPHORESIS AND COLUMN CHROMATOGRAPHY NOMENCLATURE

One problem in interpreting and reporting HPLC results is the different nomenclature used for electrophoretic and chromatographic (HPLC) analysis. Electrophoresis has been used to investigate human red cell proteins since the 1940s (Stern, Reiner & Silber, 1945; Pauling, Itano *et al.*, 1949; Huisman & Dozy, 1962; Huisman, 1986). Initially, the main adult haemoglobin band was called Haemoglobin A (Hb A) and the fetal haemoglobin band was called Haemoglobin F (Hb F). Sickle haemoglobin was discovered in 1948 (Pauling, Itano *et al.*, 1949) and was initially called Haemoglobin B, but this was soon changed to Haemoglobin S. Other haemoglobin variants discovered soon afterwards used the succeeding letters of the alphabet Hb C (Itano & Neel, 1950; Hunt & Ingram, 1960); Hb D (Itano, 1951; Baglioni, 1962); and Hb E (Chernoff, Minnich & Chongchareonsuk, 1954; Hunt & Ingram, 1961), and later, when most of the letters of the alphabet had been used up, it was decided to use the place name

of the geographical location of the propositus or of the laboratory describing the new haemoglobin (Neel, 1953; Gerald & Ingram, 1961). As stated earlier, Kunkel (Kunkel & Wallenius, 1955) recognized the additional presence of a minor haemoglobin and soon after they showed that it was raised in carriers of thalassaemia and designated it Hb A_2 (Kunkel *et al.*, 1957; Lehmann, 1957). Soon after, they noticed a second minor haemoglobin band (Kunkel & Bearn, 1957) that was designated Hb A_3 by the International Society of Hematology (Lehmann, 1957).

In the late 1950s and 1960s, column chromatography was used to investigate the heterogeneity of normal haemoglobin (Hb A and Hb F) and of some variant haemoglobins (such as Hb S and Hb C). When these analyses were undertaken, a different nomenclature was used that related to the order of elution of the haemoglobin off a cationic column eluted with a buffer of increasing pH and/or ionic strength. The first (small) peak eluting was called A_I and the second (main) peak A_{II} and a third (small) peak A_{III}. It was soon shown that Kunkel's Hb A_2 was similar to the peak labelled A_{III} and that Kunkel's Hb A_3 was similar to A_I (Schnek & Schroeder, 1961). Fetal haemoglobin was also found to consist of more than one peak on column chromatography, a small peak: F_I and a main peak: F_{II}. It was soon found that Hb A_I could be subdivided into four smaller peaks called Hb $A_{Ia,b,c}$ and d. It was found that Hb $A_{Ia,b,c}$ were all glycated (Bunn *et al.*, 1976), whereas Hb A_{Id} was formed as an ageing derivative owing to the disulphide linkage of glutathione with the β93 cysteine residue (Huisman & Dozy, 1962). As there might be confusion between Hb A_{II} and Hb A_2, Hb A_{II} was renamed Hb A_o; the total Hb A is therefore the sum of Hb A_o, Hb $A_{Ia,b,c}$ and Hb A_{Id}. The peak F_{II} was similarly renamed Hb F_o. The, usually small, Hb F_I peak has been shown to be acetylated Hb F_{II}; and because it is only a small proportion of the total Hb F, it is only readily seen where there is a large amount of Hb F such as in samples of umbilical cord blood or blood samples obtained in the neonatal period. Variant haemoglobins, such as Hb S, also subdivide in a similar manner to Haemoglobin A because of adduct formation and so the total Hb S is the sum of Hb S_o together with Hb $S_{Ia,b,c}$ and Hb S_{Id}.

POSITIVE IDENTIFICATION OF THE HAEMOGLOBIN FRACTIONS BY PRESENT-DAY (2011) COMMERCIALLY AVAILABLE AUTOMATED HPLC TECHNOLOGY

Knowledge of the behaviour and the chromatographic elution patterns is important because it can affect the putative identification of peaks, their retention times and accurate quantification; for instance, in a non-transfused homozygote for Hb S or a compound HbS/β zero thalassaemia (a person with sickle cell disease), there is no Hb A but peak(s) due to Hb $S_{Ia,b,c}$ often elute in a position similar to Hb A_o and often get labelled as Hb A_o, hence changing the apparent genotype from homozygous Hb S to Hb S/β plus thalassaemia. Also when Hb S is present, the Hb S_{Id} peak often elutes in the same place as the Hb A_2 peak leading to a falsely 'raised' concentration of Hb A_2, incorrectly suggesting that the patient has also inherited β-thalassaemia. Electrophoresis on cellulose acetate at alkaline pH will quickly elucidate the presence or absence of Hb A. On the other hand, the 'apparent' Hb A_o peak may in fact be a true finding in which case, and in the presence of microcytosis, the diagnosis could indeed be Hb S/β plus thalassaemia so long as no α-thalassaemia is responsible for the microcytosis. Therefore, although accurate identification and quantification of Hb A and A_2 have paramount importance in reaching the correct diagnosis of a large spectrum of Hb S β plus thalassaemia cases, ultimately it will be the DNA analysis that will precisely define the genotype.

Accurate quantification of Hb S levels in heterozygotes also has important implications in co-inheritance of α-thalassaemia (Higgs *et al.*, 1984). In the presence of four normally expressed α genes (αα/αα), the expected Hb S level will approximately be 38%. In cases with α plus thalassaemia heterozygosity (-α/αα), the Hb S level will generally drop to approximately 35% or less. In cases with only two functional α-globin genes in *CIS* (–/αα) or *TRANS* (-α/-α), the Hb S level will drop further down to 25%, while in Hb H disease with only one functional α-globin gene left, Hb S may drop further to around 16%. Such correlations demonstrate the power of quantification and its usefulness in reaching a putative diagnosis to be confirmed at the molecular level.

DNA analysis will confirm the nature of any haemoglobin variant, and mass spectrometry has been developed sufficiently to give accurate, detailed

analysis of haemoglobin even if only small samples (50 μL) of blood are available (Wild *et al.*, 2001; Wild, Green & Stephens, 2004).

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Several companies now produce HPLC systems. These are all similar, in that all utilize a weak cation-exchange column in their instruments. A sample of a RBC lysate is injected into the system; haemoglobin molecules will adsorb onto the column as they are charged molecules in the buffer system used. An eluting buffer is then injected into the system; the composition of this solution gradually changes by increasing the ionic strength. The haemoglobin fractions (normal haemoglobins and any haemoglobin variant) will elute off of the column when the ionic strength of the eluting solution is greater than the attraction to the column. Different haemoglobin variants will have different overall charges because of the amino acid substitution that is present. Thus, the time at which the haemoglobin molecule elutes off of the column (retention time) is characteristic and reproducible, but not unique, for each haemoglobin variant. As the haemoglobin fractions elute off the column, they pass through a detection system that utilizes absorbance readings at 415 and 690 nm. The percentage of each haemoglobin fraction (Hb A, Hb F, Hb A_2, and any variant) is calculated by summing the area under each peak in the chromatogram. Glycosylated haemoglobins (such as Hb A_{Ic}) and oxidized methaemoglobin elute from the column as separate peaks distinct from and before Hb A. Fast variants, such as Hb H or Hb Barts may not be quantified as they usually elute off the column before the instrument begins to integrate with many systems designed for adult samples but are usually quantitated in systems designed for neonatal samples (Van Delft *et al.*, 2009).

Automated HPLC has four advantages over the manual methods.

- Many samples can be loaded at a time, and if necessary, their analysis can continue overnight in the absence of the analyst.
- A much smaller sample size is required, usually of the order of 50 μL compared with 1–2 mL for the manual methods.
- Not only Hb A_2 but also Hb F and any other common haemoglobin variants present (such as Hb S, C, D-Punjab, E or O-Arab) can be separated and quantitated in the same run.
- The equipment can usually be operated by relatively junior staff but the interpretation and QC require considerable experience.

However, dedicated automated HPLC may also have some disadvantages compared with the manual methods. First, as stated earlier, the column/buffer combination is sometimes optimized for Hb A_{Ic} by compressing the final part of the chromatogram, and this may mean that the Hb A_2 peak does not separate adequately from the Hb A_o peak and also that some Hb A_2 variant peaks are missed. Second, it requires analysts with considerable experience of column chromatography and interpretation of the chromatograms. Some laboratories may prefer to prepare their own columns and buffers and in that situation, it is their responsibility to ensure adequate separation, labelling and integration of the various haemoglobin peaks.

WHEN INTERPRETING HPLC CHROMATOGRAMS CHECK

- That controls elute with the retention time (in the 'window') expected. If they do not do so, it is likely that the buffer/column temperature is incorrect, although other causes are possible such as an incorrect buffer, column or flow rate, or dirty tubing or an exhausted column.
- That the peaks are symmetrical. If they are asymmetrical, a variant may be present or the tubing or column may be contaminated or in a bad condition.
- Check that all the major and minor peaks expected are present and check to see whether any unexpected major or minor peaks are present.
- If accurate quantification is required, check that the baseline is flat for the whole of the chromatogram and that the peak is completely separated from other peaks and reaches the baseline on both sides.

CAPILLARY ZONE ELECTROPHORESIS

This method utilizes a thin capillary made of fused silica with an outer coating of polyimide, usually with an inner diameter of 50 or 75 μm. Because the capillary has a very large surface-to-volume ratio, it is excellent at dissipating the heat generated by the

applied voltage. Thus, very large voltages (10–30 kV) can be used, and because of these high voltages, the run times are significantly shortened and the resolution increased. The inner surface of the capillary tube has a negative charge because of the bare silica. When an electric field is applied, the buffer solution within the capillary generates an electro-endosmotic flow (EOF) that moves towards the cathode. A sample of haemolysate is injected into the system, and the electric current applied causes separation of individual haemoglobins because of differences in overall charges. Haemoglobin variants, if present, may separate because of a charge difference resulting from the amino acid substitution. However, regardless of the overall charge of each haemoglobin fraction, the EOF is still stronger than any attraction to either pole, and all haemoglobin fractions will move towards the cathode. All the haemoglobin molecules move past a detector that measures the absorbance at 415 nm. An electropherogram (similar to a chromatogram) is thus generated; the percentage of each haemoglobin fraction (Hb A, Hb F, Hb A_2 and any haemoglobin variants) is calculated (but see the paragraph above on peak integration as 'spatial areas' have to be used for quantification because the fractions pass the detector at different velocities). For haemoglobinopathy work, capillary zone electrophoresis has the same advantages as HPLC over manual methods and some equipment and reagents also have an advantage over HPLC in that haemoglobin adducts such as glycated $X_{Ia,b,c}$ and the ageing glutathione adduct, X_{Id}, do not separate from the main haemoglobin peaks, and this makes interpretation easier than with HPLC. Some variant haemoglobins (Table 6) migrate with or very close to Hb A_2 and therefore interfere with the quantification of Hb A_2.

CAPILLARY ISOELECTRIC FOCUSSING

In cIEF, the electro-osmotic flow is minimized by coating the inside of the capillary with a solution to neutralize the silanols. The capillary is filled with a mixture of ampholytes and haemolysate. After application of the voltage for a period of time, the haemoglobins become focussed at their pI value. They are then mobilized and pass through the detector operating at 415 nm (Hempe & Craver, 1999). An electropherogram (similar to a chromatogram) is thus generated; the percentage of each haemoglobin fraction (Hb A, Hb F, Hb A_2 and any haemoglobin variants) is calculated.

ELECTROPHORESIS AND ELUTION

This gives satisfactory results in experienced hands if undertaken as stated in the 1978 Recommendations (International Committee for Standardization in Haematology, 1978).

MICROCOLUMN CHROMATOGRAPHY

This gives satisfactory results in experienced hands if undertaken as stated in the 1978 Recommendations (International Committee for Standardization in Haematology, 1978).

CONCLUSIONS

- It is essential to be able to measure the proportion of Hb A_2 in a red cell haemolysate in order to make, or exclude, the diagnosis of β-thalassaemia trait.
- As there may be only a small numerical difference in Hb A_2 levels between people who have β-thalassaemia trait and those who do not, precision and accuracy are both important. However, excluding beta-thalassaemia, because the Hb A_2 level is 0.2% below the cut-off is not recommended, and one should always include all haematological parameters in reaching a conclusion, and in case of doubt, DNA analysis should be undertaken.
- Methods used in clinical laboratories first involve separating the Hb A_2 from Hb A and any other haemoglobin present and then quantitating the different fractions present.
- Historical methods of analysis (cellulose acetate electrophoresis with elution or microcolumn chromatography) that were discussed in a previous publication (International Committee for Standardization in Haematology, 1978) can produce results that are satisfactory for clinical purposes but are time-consuming and need technical expertize.
- Automated HPLC with dedicated commercially available buffers and columns is increasingly being introduced and can give satisfactory results when no haemoglobin variant is present. However, when haemoglobin variants are present, experience is

necessary to interpret the chromatograms. It is essential that the reagents are optimized for Hb A_2 rather than Hb A_{Ic} and that the baseline is appropriate.

- Automated capillary electrophoresis and automated capillary isoelectric focussing with commercially available capillaries and buffers are becoming available and can also produce satisfactory results if no haemoglobin variant is present, but experience is necessary to interpret the electropherograms.
- At present, the only internationally recognized standard for Hb A_2 is the WHO first Reference Material (89/666) that is available from NIBSC, Blanche Lane, South Mimms, Potters Bar, Hertfordshire, EN6 3QG, UK (http://www.nibsc.ac.uk). It is hoped that in the foreseeable future, a new certified reference material will be available from the European Commission Joint Research Centre, Institute for Reference Materials and Measurements.

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