



ICSH recommendations for the measurement of Haemoglobin F

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doi:10.1111/j.1751-553X.2011.01367.x

Received 13 April 2011; accepted for publication 13 June 2011

Keywords

Haemoglobin F, high-performance liquid chromatography, capillary electrophoresis, recommended methods, haemoglobinopathy, thalassaemia

SUMMARY

Measurement of the Haemoglobin F in red cell haemolysates is important in the diagnosis of $\delta\beta$ thalassaemia, hereditary persistence of fetal haemoglobin (HPFH) and in the diagnosis and management of sickle cell disease. The distribution of Hb F in red cells is useful in the diagnosis of HPFH and in the assessment of foeto-maternal haemorrhage. The methods of quantifying Hb F 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 ICSH recommendations were published (International Committee for Standardization in Haematology, 1979) concerning the quantification of Haemoglobin F (Hb F); and during this time, there have been several new analytical developments in the field, and therefore the ICSH Board consider that the original recommendations should be revised. Hb F is a haemoglobin tetramer composed of two α and two γ globin chains ($\alpha_2\gamma_2$). It is a normal haemoglobin and is the major haemoglobin present in the fetus but is gradually replaced after birth by Hb A ($\alpha_2\beta_2$) as the γ chains are replaced by β chains. The measurement of Hb F in red cells is clinically useful in the diagnosis of $\delta\beta$ thalassaemia because the amount of Hb F is raised in this condition. Hb F is also slightly raised (1–5%) in pregnancy (Pembrey, Weatherall & Clegg, 1973), in hereditary persistence of fetal haemoglobin (HPFH) and sometimes in β thalassaemia trait. It is occasionally raised in other situations (Table 1), but apart from sickle cell disease and feto-maternal haemorrhage (FMH), there is little clinical utility in measuring the Hb F in these other situations (Mosca *et al.*, 2009). The clinical effects of sickle cell disease can be reduced by using drugs such as hydroxyurea (hydroxycarbamide) which raise the Hb F level and measuring the Hb F level can then be clinically useful in helping to determine the appropriate dose regime. The

Table 1. Conditions in which Hb F is raised

Physiological
Neonates
Pregnancy
Hereditary
$\delta\beta$ thalassaemia
β thalassaemia major and intermedia
β thalassaemia trait (sometimes)
Hereditary persistence of fetal haemoglobin
Sickle cell anaemia \pm treatment with
hydroxycarbamide (hydroxyurea)
Unstable β chain variants
Acquired (Hb F sometimes raised)
Recovery from bone marrow hypoplasia
Leukaemia
Myelodysplasia
Thyrotoxicosis
Hepatoma

Hb F, Haemoglobin F.

assessment of the proportion of red cells of fetal origin is clinically useful in assessing the amount of FMH that has occurred and the medication to be given. Assessing the proportion of cells that contain a high proportion of fetal haemoglobin (often called F cells) is also useful in the diagnosis of 'classical' deletional HPFH.

ANALYTICAL TECHNIQUES

In 1866, it was noted that fetal haemoglobin is more resistant to alkali than adult haemoglobin (von Korber), but it was not until 1951 (Singer, Chernoff & Singer, 1951) that this property was utilized to measure the amount of fetal haemoglobin present in red cell haemolysates (the one-minute alkali denaturation technique). This technique was refined by in 1959 (Betke, Marti & Schlicht, 1959) when they published their two-minute alkali denaturation technique and this was further modified in 1972 (Pembrey, Mcwade & Weatherall, 1972). This two-minute alkali denaturation technique has been considered to give accurate and clinically useful results in the 0–40% range of Hb F but to underestimate the amount of Hb F at higher levels; although in clinical situations, this is not a problem. This method was selected by the ICSH as the recommended method for clinical use (International Committee for Standardization in Haematology, 1979), and in the same article, a method was described for preparing a standard for Hb F. The method for producing a Hb F standard was further modified by Wild (1986) to produce the 1st WHO International Reference Material for Hb F (World Health Organization Expert Committee on Biological Standardization, 1994) for use with the two-minute alkali denaturation method. This is a stabilized, freeze-dried cyanmethaemoglobin preparation prepared from blood obtained from the father of a patient with beta thalassaemia major and is held at National Institute of Biological Standards and Controls (NIBSC) South Mimms, Herts, UK.

In 1958, column chromatography of haemolysates from human blood showed that fetal haemoglobin could be separated from adult haemoglobin and that a small proportion of the fetal haemoglobin separated from the main Hb F peak (Allen, Schroeder & Balog, 1958), and it was later shown that this small peak was acetylated Hb F (Schroeder *et al.*, 1962). Because the acetylated Hb F eluted before the main peak, it was called F_I and the main peak F_{II}, but the main

peak was later renamed F₀ when the main peak of adult haemoglobin was renamed A₀. It was later shown that these two types of fetal haemoglobin could also be separated by isoelectric focusing (Basset *et al.*, 1978). Automated, dedicated high-performance liquid chromatography (HPLC) was introduced in the late 1980s to simplify and speed up screening of maternal blood samples for Hb A₂, Hb F and common haemoglobin variants (such as Hb S, C, D, E and O). Recently, automated capillary zone electrophoresis (CZE) has been introduced as an alternative to automated HPLC for screening blood samples for Hb A₂, Hb F and the common variant haemoglobins (Van Delft *et al.*, 2009). The γ -chains of Hb F may contain either glycine or alanine at position 136 (γ^G and γ^A) because of the presence of two γ genes (γ^G and γ^A) coding for almost identical products, but these two forms do not separate on HPLC or CZE, but at the present time, there is no clinical advantage in measuring the γ^G & γ^A chains separately. A radial immunodiffusion technique has also been developed and is commercially available. With the huge increase in throughput of Hb A₂ and Hb F measurements in hospital laboratories because of both the mandatory and the voluntary screening programmes, automated HPLC or CZE have become the main tool to quantify Hb F and Hb A₂ in most laboratories in Europe and North America and is being increasingly used in Asia and Africa, and capillary isoelectric focusing (cIEF) is also being introduced. However, dedicated instruments are available from different companies, and these approach the analytical issues in different ways.

When it is necessary to assess the proportion of red cells that are of fetal origin either a cytochemical technique such as the Kleihauer method (Kleihauer, Braun & Betke, 1957; Kleihauer, 1974) or the Shepard modification of it (Shepard, Weatherall & Conley, 1962) or flow cytometry can be used (Nelson *et al.*, 1998; Leers *et al.*, 2007). These techniques can also be used to study cases of HPFH.

PEAK QUANTIFICATION

With automated systems such as HPLC, CZE and cIEF, the accuracy of peak quantification is dependent on both the 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 is kept within the linear range because if too much sample is applied any large peaks may be underestimated leading to a relative overestimation of small peaks such as the Hb F. It is equally important that the baseline used for the integration is correct as only those parts of the peak that are above the baseline will be quantified, 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 quantifying Hb F, 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 as 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 overestimation of the Hb F 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 F. For all these reasons, it is extremely important that the chromatogram, or electropherogram, is inspected carefully before the results are authorized. Because Hb F is reported as a ratio of Hb F 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 capillary isoelectric focusing it is the peak areas that are related to the amount of haemoglobin present, it should be noted that in CZE the peak areas should not be used for quantification because of the different migration velocities through the detector, instead the 'spatial area' (integrated area divided by the migration time) should be used (Huang, Coleman & Zare, 1989; Hempe & Craver, 1999).

SAMPLE COLLECTION AND STORAGE

Any anticoagulant can be used although it is common to use K₂EDTA which is the anticoagulant used for the analysis of blood counts. Ideally, the analysis should be undertaken as soon as possible after venesection, but storage of a sealed whole blood sample at 4 °C for 2–3 weeks is acceptable 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 because considerable denaturation of haemoglobin occurs if a sample is kept at 50 °C for more than 1 h. Sample stability after freezing at –20 °C 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 in liquid nitrogen. Thawing and re-freezing of blood samples should be discouraged. A partially coagulated blood sample should not be accepted for Hb F quantification by automated equipment as it may contaminate or block the tubing.

EXPRESSION OF RESULTS

To be clinically useful, the results of the Hb F quantification have to be expressed as a percentage of the total haemoglobin (which will usually include Hb A, Hb F, Hb A₂ and any glycated, acetylated or aged adducts).

PRECISION AND ACCURACY

As stated earlier, both precision and accuracy are extremely important because a raised level of Hb F may be indicative of the diagnosis of Hb F or delta beta thalassaemia trait. The precision obtainable in the manual techniques is ±0.1% in the final answer that is equivalent to an SD of 0.05% (leading to a CV of 5% in the normal range and better when raised as often occurs in thalassaemia). A similar precision should be obtainable in modern automated HPLC and capillary electrophoretic equipment and is sufficient for diagnostic purposes. Some HPLC systems are not able to detect Hb F below 1% but because 1% is the normal

cut-off in adults, this is not a problem. Nevertheless, CVs of the order of 2–3% can be obtained at higher Hb F concentrations (Papadea & Cate, 1996; Paleari *et al.*, 2005). Accuracy is harder to establish and monitor but comparison with National or Regional quality assessment schemes can be helpful. A WHO reference material is available, but it was only analysed by the two-minute alkali denaturation technique.

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

Several companies now produce dedicated HPLC systems (Van Delft *et al.*, 2009). These are all similar in that they all utilize a weak cation-exchange column in their instruments. A sample of RBC lysate is injected into the system; haemoglobin molecules will attach to the column as they are charged molecules in the buffer system used. An eluting solution is then injected into the system; the composition of this solution changes by increasing the ionic strength. The haemoglobin fractions (normal plus any haemoglobin variant) will elute off the column when the ionic strength of the eluting solution is greater than the retention 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 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 which utilizes absorbance readings at 415 and 690 nm. The percentage of each haemoglobin fraction (Hb A, Hb F, Hb A₂, and any variant) is calculated by summing the area under the curve of each peak of the chromatogram. Glycated haemoglobins, such as Hb A_{1c}, and methaemoglobin elute from the column as separate peaks distinct from and before Hb A as does the 'aged peak' A_{1d}. Fast variants, such as Hb H or Hb Barts, may not be quantified as they may elute off the column before the instrument begins to integrate in many systems designed for adult samples, and this will affect the quantification of Hb F. However, they are usually quantified in systems designed for neonatal samples that start integrating sooner.

These instruments can measure Hb F with sufficient accuracy for clinical purposes as long as the Hb F peak is adequately separated from the other

peaks and from any variant haemoglobins present. Because the F_1 peak is only a very small proportion of the total Hb F, it is only apparent on neonatal samples, or adult samples with fairly large amounts of Hb F. If an alpha globin variant is present that separates from Hb A, then there will be a Hb F variant that will often separate from normal Hb F but it may not separate from the other haemoglobin adducts present and then the total Hb F will not be adequately quantified. Hb F variants may also be due to mutations in the γ globin chain, and again this may result in a separate peak and incorrect quantification. Some β -chain variants and/or their adducts will not separate from Hb F, and this will also lead to incorrect quantification. In the absence of a haemoglobin variant, the Hb F separates from Hb A, its glycosylated adducts and the Hb A₂, and therefore its quantification should be satisfactory. The presence of any haemoglobin variant eluting before Hb A₀ on HPLC will frequently lead to inaccurate quantification. In these situations, a different technique such as the two-minute alkali denaturation method will give a clinically useful result.

CAPILLARY ZONE ELECTROPHORESIS

This method utilizes a thin capillary made of fused silica with an outer coating of polyimide and 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-osmotic 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, 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 which mea-

sures 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 A₂, plus any haemoglobin variants) is calculated (but see the paragraph aforementioned on peak integration because 'spatial areas' have to be used for quantification because of the different velocities of peaks past the detector). For haemoglobinopathy, work CZE has the same advantages as HPLC over manual methods but some equipment and reagents have an advantage over HPLC in that haemoglobin adducts such as glycosylated $X_{1a,b,c}$ and the ageing glutathione adduct, X_{1d} , do not separate from the main haemoglobin peaks, and this makes interpretation easier than with HPLC.

CAPILLARY ISOELECTRIC FOCUSING

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), and an electropherogram (similar to a chromatogram) is thus generated; the percentage of each haemoglobin fraction (Hb A, Hb F, Hb A₂, plus any haemoglobin variants) is calculated.

CYTOCHEMICAL TECHNIQUE

For semi-quantitative estimation of the distribution of fetal Hb in the red cell population. A blood smear is prepared on glass slide, fixed in 80% ethanol and then immersed for 5 min at 37 °C in citrate-phosphate buffer pH 3.3 (citric acid 0.075 M and disodium phosphate 0.05 M). The elution time and pH of the buffer have to be carefully controlled (Shepard, Weatherall & Conley, 1962; Kleihauer, 1974). In patients with deletional HPFH, all RBCs show almost uniform retention of haemoglobin (pancellular distribution), whereas in β thalassaemia trait with increased Hb F, and nondeletional HPFH, a heterocellular distribution of haemoglobin occurs.

FLOW CYTOMETRY

Flow cytometric assay can be used to assess the number and distribution of 'F cells' in the blood.

Adult F cells are erythrocytes where the haemoglobin is represented by a mixture of Hb A and Hb F (Wood *et al.*, 1975). All individuals have some F cells estimated at an average of 2.7% of cells in normal adults by an earlier nonflow cytometric fluoresceinated antibody technique (Davis *et al.*, 1998). Flow cytometric techniques suggest that this level may be even higher in normal individuals (Chen, Bigelow & Davis, 2000). F cells can be more prevalent in anaemia and in some haemoglobinopathies. The enumeration of 'light pink'-staining F cells vs. 'deep red'-staining fetal cells by the traditional Kleihauer slide test may be difficult (CLSI, 2001). Quantification of F cells may be helpful in the management of patients with sickle cell disease treated with hydroxyurea and also when monitoring patients with myelodysplasia. However, measurement of the total Hb F in a haemolysate by HPLC or CZE is usually easier and clinically just as useful. Commercial assays have been developed for undertaking flow cytometric FMH assays.

As is the case with all clinical assays, the use of internal quality control is important. Controls cannot only be used to assess the quality of Kleihauer staining but are also used to better define the region of analysis positive for fetal cells in flow cytometry. Controls can be made locally in the laboratory, using artificial mixtures of fresh cord blood and adult blood samples, or a commercial preassayed product can be used. Quality performance of the FMH or fetal red cell detection assays are described in the CLSI H52-A document (CLSI, 2001). This lists potential problems with the Kleihauer and flow cytometry methods.

TWO-MINUTE ALKALI DENATURATION TECHNIQUE

If a manual technique is required, the two-minute alkali denaturation method (Betke, Marti & Schlicht, 1959) as modified by Pembrey, Mcwade and Weatherall (1972) is still a useful way of assessing the Hb F concentration in clinical situations but has the disadvantage that it is time consuming and requires experience to obtain good results.

RADIAL IMMUNODIFFUSION TECHNIQUE

Haemoglobin F can be quantified by radial immunodiffusion. Plates containing anti-Hb F are available

commercially with associated standards covering the range 0.5–10% Hb F.

INTERPRETATION OF RAISED LEVELS OF Hb F

At birth, the average level of Hb F is around 80% but depends on the age of gestation. After birth, the Hb F steadily decreases to reach adult levels (<1%) during the second year of life. In thalassaemia trait levels of up to 10% may be recorded depending on the mutation and the haplotype. In $\delta\beta$ thalassaemia trait levels from 5% to 20% are found. In HPFH heterozygotes, Hb F levels from 2.5% in the 'Swiss' type and to up to 30% in the deletional forms are found. In homozygous deletional HPFH and in homozygous nontransfused β^0 thalassaemia major, the Hb F level will be 100%. Erythropoietic stress and malignancies may induce variable levels of Hb F (see Table 1).

CONCLUSIONS

It is important to be able to quantify Hb F for the diagnosis of $\delta\beta$ thalassaemia and HPFH and in sickle cell disease at diagnosis, and whilst monitoring the effect of hydroxycarbamide (hydroxyurea) in sickle cell disease or β thalassaemia major or intermedia. The accuracy and precision of Hb F quantification in blood samples with Hb F levels in, or very close to, the normal range is often poor, but this is rarely a diagnostic problem. Hb F levels of 2–3% calculated in HPLC areas crowded with glycated fractions should be critically examined for overestimation. Assessment of the cellular distribution is useful in the diagnosis of HPFH and when investigating suspected FMH. Methods used in clinical laboratories first involve separating the Hb F from Hb A and Hb A₂ and from any other haemoglobin present and then quantifying the different fractions and calculating the proportions of the different haemoglobins because it is the proportion, not the absolute amount, of Hb F that is clinically important. Historical methods of analysis. The two-minute alkali denaturation technique (Pembrey, Mcwade & Weatherall, 1972; International Committee for Standardization in Haematology, 1979) which were discussed in a previous publication can produce results that are satisfactory for most clinical purposes but are time consuming and need experience to

obtain good results. Automated HPLC with dedicated commercially available buffers and columns are increasingly being introduced and can give satisfactory results if no haemoglobin variant is present, but considerable experience is necessary to interpret the chromatograms. If any haemoglobin variants are present, the analysis may lead to misleading results. It is essential that the reagents are optimized for Hb F rather than Hb A_{1c} and that the baseline is appropriate. Automated capillary electrophoresis and automated capillary isoelectric focusing with commer-

cially available capillaries and buffers are becoming available and can also produce satisfactory results if no haemoglobin variant is present, but considerable experience is necessary to interpret the electropherogram. At present, the only internationally recognized standard for Hb F is the WHO 1st Reference Material (85/616) that is available from NIBSC, Blanche Lane, South Mimms, Potters Bar, Hertfordshire EN6 3QG, UK (<http://www.nibsc.ac.uk>), but it was only analysed by the two-minute alkali denaturation technique.

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